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The Journal of Laboratory and Clinical Medicine

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

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Vol. 38, No. 5, November, 1951. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription Price: United States, its possessions, Pan-American Countries, $10.00 per year; Canada, $11.00; Foreign, $11.00. Subscriptions to medical students, interns and residents, half price. Entered as Second-Class Matter, October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

LOCALIZATION OF RADIOACTIVE *l*-THYROXINE IN THE NEUROHYPOPHYSIS

J. M. Jensen, M.D.,* and D. E. Clark, M.D.
Chicago, Ill.

In 1932 Schittenhelm and Eisler[1] published findings which seemed to indicate that a relationship existed between thyroxine supply and iodine content of specific parts of the central nervous system. Their experiments were performed on dogs, cats, and rabbits. The experimental animals received intravenously 1.0 mg. of thyroxine per kilogram of body weight, and after a given time interval were sacrificed by exsanguination. Iodine determinations were done by the von Fellenberg method[2] on various parts of the brain. The results reported are interesting in that the tuber cinereum and midbrain showed a tenfold increase in iodine concentration, while other parts of the brain remained at or near control, noninjected levels.

Schittenhelm and Eisler[3] subsequently established normal iodine values for the brain tissues of man and animals. Consistently, the highest concentration of iodine was found in the midbrain and tuber cinereum. Normal iodine values determined from the brains of healthy animals were compared with those from animals prepared with thyroxine and from animals previously subjected to thyroidectomy. The results showed that alterations of iodine concentration induced by these procedures were confined to the tuber cinereum and midbrain. Removal of the thyroid gland resulted in the iodine content of the midbrain being equal to or below that of other parts of the brain.[3]

On the basis of these findings, Schittenhelm and Eisler hypothesized that thyroxine or its metabolic product was selectively concentrated in the midbrain and tuber cinereum. They proposed that a direct connection existed between thyroid gland activity and the iodine content of specific parts of the central nervous system.

This hypothesis, and particularly the experiments on which it is based, may be significant. Many steps in the regulation of thyroid function remain unsatisfactorily explained. The hormonal mechanism postulated for the pituitary-thyroid axis is incompletely understood. Many investigators have speculated on the role of the hypothalamus in the regulation of thyroid physiology, but direct evidence has been lacking.[4-6]

From the Department of Surgery of the University of Chicago.

This work was aided in part by the Charles H. and Mary F. S. Worcester Memorial Fund, by an institutional grant from the American Cancer Society, and by a grant from the United States Public Health Service (National Cancer Institute).

The radioactive iodine used in this investigation was supplied by the Oak Ridge National Laboratories, Oak Ridge, Tenn., and was obtained under allocation from the United States Atomic Energy Commission.

The radioactive thyroxine used in this investigation was prepared by the Radioactive Pharmaceutical Department, Abbott Laboratories, North Chicago, Ill.

Received for publication July 16, 1951.

*Submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine with Honors.

The concept that thyroxine may be selectively localized in the hypothalamus has remained controversial, primarily because of the difficulty in determining the extreme small quanities of iodine involved. The von Fellenberg method,[2] utilized in all determinations by Schittenhelm and Eisler, employs strong oxidation and ashing of the tissues followed by titration with thousandth-normal thiosulfate solution. The accuracy of this method has been questioned, and especially when applied to a tissue high in lipoid such as the central nervous system.[7] More modern techniques of determining iodine in biological material are based on the reducton of ceric ions to cerous ions by arsenious acid. These appear to be reliable for serum protein-bound iodine, but contradictory results have been reported for brain tissue.[7, 8]

The present investigation was undertaken because it seemed likely that radioactive *l*-thyroxine, if selectively localized in any part of the brain, could be identified by counting and radioautographic techniques.

METHODS

Radioactive *l*-thyroxine was prepared by an exchange reaction substituting radioactive iodine (I^{131}) into the 3′, 5′ positions of the thyroxine molecule. The specific activity of each batch was determined before shipment, and the preparation was shown to be free from iodine, iodate, and iodide.[9]

Eight adult rabbits were used in this experiment. Five of these were normal animals. Three animals had previously each received 12 mc. of radioactive iodine over a six-week period, and were shown by serum precipitable iodine determinations to be hypometabolic. Such determinations are considered to be a reliable index of thyroid gland activity.[10]

Four animals received radioactive iodine but no thyroxine. Three of these were normal, and one was hypometabolic. Four animals were given radioactive *l*-thyroxine. Two of these were normal and two were hypometabolic. All material was given intravenously.

The animals were anesthetized with sodium pentobarbital four hours after receiving the radioactive material. The carotid artery and internal jugular vein of each animal were isolated bilaterally. Four of the animals were perfused through these vessels with a solution of physiological saline followed by a solution of formalin. Perfusion was undertaken in an attempt to partially fix the brain in situ and to wash radioactive material from some of the larger vessels. Four of the animals were not perfused; these were killed by exsanguination.

The brains were carefully removed with the hypophysis and hypophyseal stalk intact. This dissection was found to be expedited by an orbital, inferior approach. The meninges were removed, and the brains washed in neutral formalin solution.

Blocks of tissue, each about 3 to 4 mm. in thickness, were cut transversely through the brain at arbitrarily chosen levels. These levels are illustrated in Fig. 1. An attempt was made to cut the blocks so that those from one specimen could be compared with those from another. Each block was placed in a small porcelain capsule and counted by a Geiger-Müller tube and scaling unit. It is felt that this procedure provides a gross estimate of the radioactivity in the tissue counted.

The blocks of tissue, after counting, were fixed and dehydrated. Several fixing and dehydrating methods were investigated to insure that the method chosen[11] would not remove significant amounts of radioactivity from the tissues. The tissues were embedded in paraffin and serial sections were cut from each block. The mounted sections were placed in close approximation with an NTB type nuclear track plate so as to produce a contact radioautograph. Selected sections from each block were floated directly onto the nuclear track plate, a method providing accurate localization of the radioactive material in the tissues.[12] The

photographic plates were exposed for twenty-one days, developed, the tissues stained with hematoxylin and eosin, and the radioautographs evaluated by gross and microscopic examination.

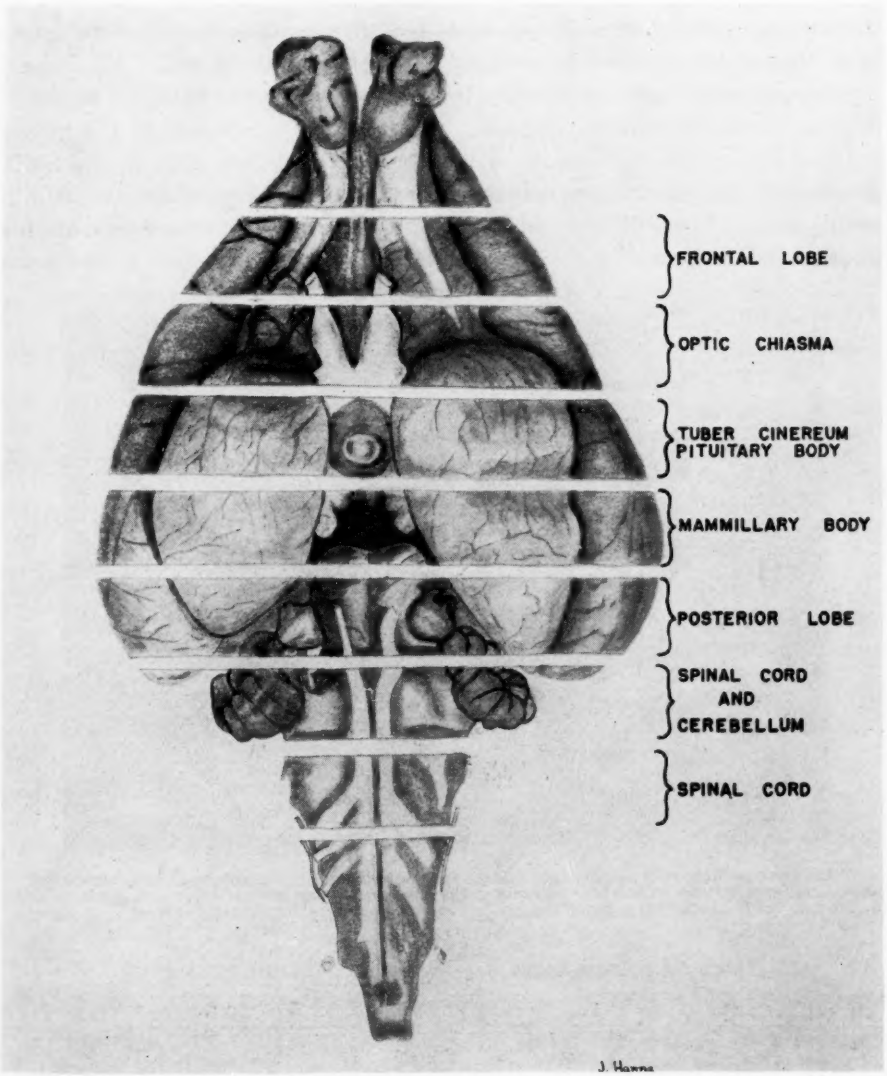

Fig. 1.—Shows levels at which blocks of tissue were cut for counting and serial sectioning.

RESULTS

Rabbits Given Radioactive I^{131}.—Four animals, one of which was hypothyroid, each received 1.0 mc. of carrier free radioactive I^{131}. Two of the animals were perfused and two were not. The tissue block counts were slightly higher in the two animals not perfused. Table I sumarizes the radioactivity in the various tissue blocks. There did not appear to be any significant localization. The radioautographs were entirely negative.

Rabbits Given Radioactive l-Thyroxine.—Four animals (Table II) received radioactive *l*-thyroxine. The dose for each animal was essentially the same. Two were normal animals and two were hypometabolic. Two were perfused and two were not. The tissue block counts from these animals were considerably higher than those from the animals receiving radioiodine alone.

The greatest radioactivity was found in the blocks from the region of the tuber cinereum and hypophysis. The radioautographs from the animals receiving radioactive *l*-thyroxine showed a selective localization in the medial eminence of the tuber cinereum, the neural lobe of the pituitary, and the infundibulum. This is illustrated in Figs. 2, 3, 4, and 5. The localization of radioactive *l*-thyroxine in the anterior lobe was much less than in the neuro-

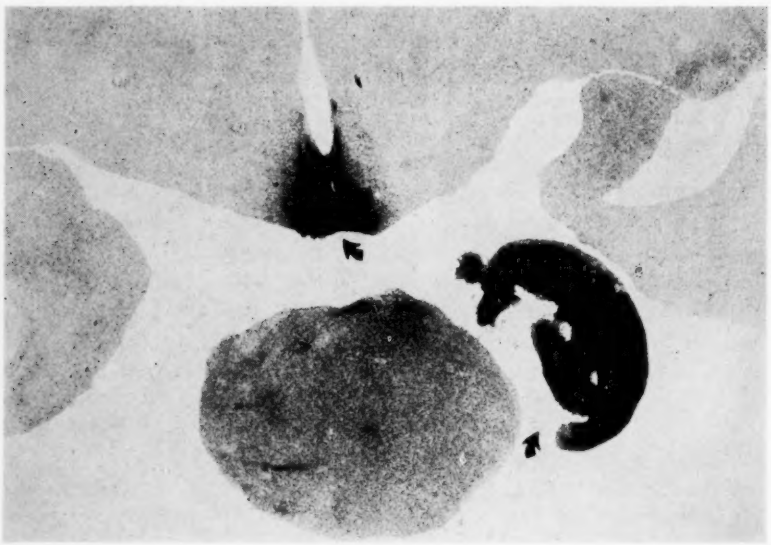

Fig. 2.—Contact radioautograph showing localization of radioactive *l*-thyroxine in the median eminence of the tuber cinereum and the neural lobe of the pituitary (arrows). Eastman Kodak NTB plate, 10 μ emulsion.

TABLE I. RABBITS GIVEN RADIOACTIVE I^{131} BUT NO THYROXINE

ANIMAL NUMBER	1	2	4	9*
Preparation given	I^{131}	I^{131}	I^{131}	I^{131}
Millicuries given	1.0 mc.†	1.0 mc.	1.0 mc.	1.0 mc.
Perfusion	275 c.c.	600 c.c.	None	None
Activity of blocks Level of:				
Tuber cinereum	2.6 c.p.s.‡	0.5 c.p.s.	7.4 c.p.s.	7.0 c.p.s.
Optic chiasm	2.0 c.p.s.	0.2 c.p.s.	4.6 c.p.s.	6.3 c.p.s.
Mamillary body	1.3 c.p.s.	0.5 c.p.s.	7.2 c.p.s.	5.5 c.p.s.
Frontal lobes	2.2 c.p.s.	0.4 c.p.s.	1.9 c.p.s.	2.3 c.p.s.
Posterior lobes	2.0 c.p.s.	–	–	5.5 c.p.s.
Cerebellum	–	0.4 c.p.s.	2.6 c.p.s.	4.3 c.p.s.
Spinal cord	1.7 c.p.s.	0.2 c.p.s.	–	–

*Hypometabolic animal.
†Oak Ridge Standard.
‡Activity expressed in counts per second. (Corrected for background.)

hypophysis (Figs. 2 and 4), but somewhat more than in the brain and spinal cord (Fig. 6).

There was no apparent difference in the degree of uptake, under the conditions of this experiment, between the normal animals and the hypometabolic animals. No localization was found in the blood vessels, meninges, or ventricles. The tissue block counts were higher in the perfused animals.

DISCUSSION

The mechanism regulating the rate of secretion of thyroid hormone has not been entirely clarified. Considerable evidence has accumulated to show

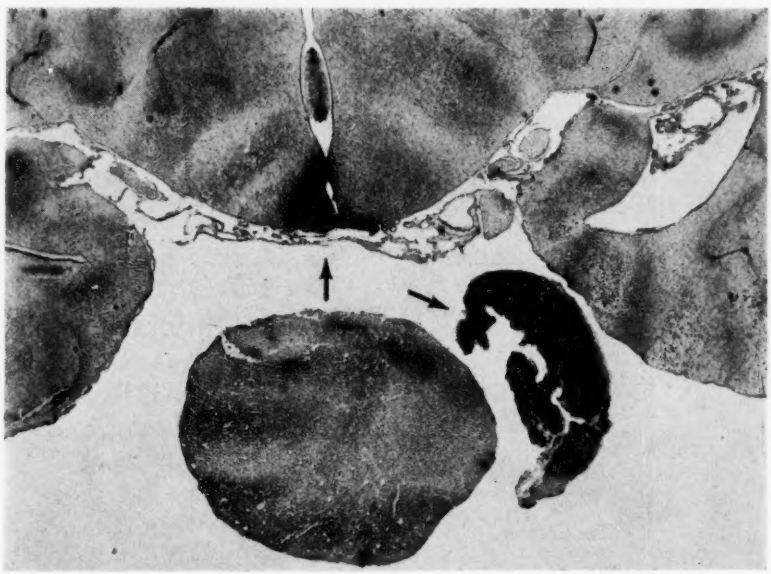

Fig. 3.—Flotation type radioautograph showing localization of radioactive *l*-thyroxine in the median eminence of the tuber cinereum and the neural lobe of the pituitary (arrows). Eastman Kodak NTB plate, 10 μ emulsion.

TABLE II. RABBITS GIVEN RADIOACTIVE *l*-THYROXINE

ANIMAL	7*	8	10*	11
Preparation given	*l*-Thyroxine 3.2 mg.	*l*-Thyroxine 3.3 mg.	*l*-Thyroxine 3.0 mg.	*l*-Thyroxine 3.0 mg.
Millicuries given	1.4 mc.	1.4 mc.	1.1 mc.	1.0 mc.
Perfusion	100 c.c.	100 c.c.	None	None
Activity of blocks Level of:				
Tuber cinereum	156.0 c.p.s.†	170.0 c.p.s.	140.0 c.p.s.	91.0 c.p.s.
Optic chiasm	113.0 c.p.s.	130.0 c.p.s.	87.0 c.p.s.	44.0 c.p.s.
Mamillary body	120.0 c.p.s.	137.0 c.p.s.	118.0 c.p.s.	57.0 c.p.s.
Frontal lobes	74.0 c.p.s.	81.0 c.p.s.	75.0 c.p.s.	33.0 c.p.s.
Posterior lobes	97.2 c.p.s.	--	119.0 c.p.s.	55.0 c.p.s.
Cerebellum	79.3 c.p.s.	107.2 c.p.s.	75.0 c.p.s.	35.0 c.p.s.
Spinal cord	--	--	--	--

*Hypometabolic animal.

†Activity expressed in counts per second. (Corrected for background.)

that the rate of thyroid hormone secretion is delimitated only through the mediation of thyrotropic hormone from the basophile cells of the anterior pituitary. If this be true, then one is confronted with the problem of what governs the production of the thyroid stimulating hormone. The most widely accepted hypothesis based on the work of Uotila[13] and others has been that the thyrotropic function is controlled in part by the level of circulating thyroid hormone. This has been assumed to act directly on the anterior lobe with little or no modifying action by the neurohypophysis.

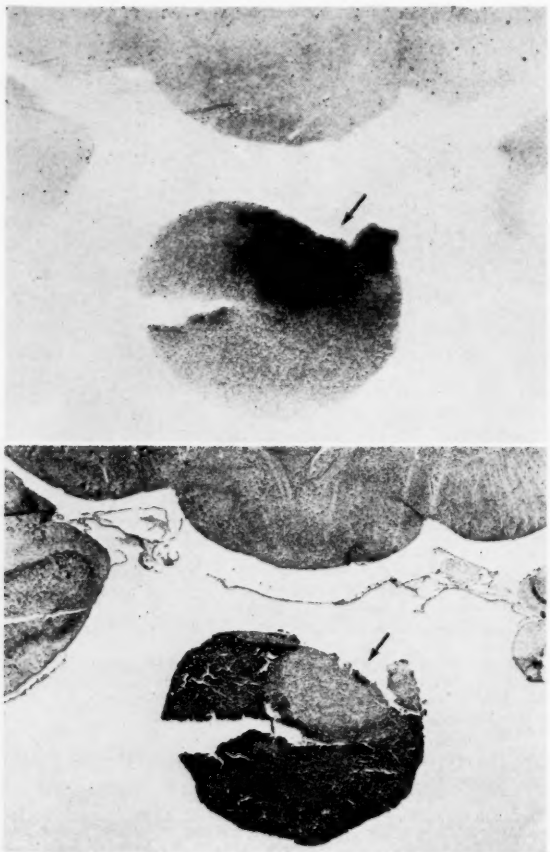

Fig. 4.—Photomicrograph (below) and corresponding radioautograph (above) showing localization of radioactive *l*-thyroxine in the neural lobe (arrows).

The demonstration of a rapid and selective localization of radioactive *l*-thyroxine in the neurohypophysis suggests that it may be regulatory in the production of thyrotropic hormone. Heinbecker[14] found that the prolonged administration of whole posterior pituitary hormone to normal dogs resulted in an increase in the basophile cells of the anterior pituitary. He concluded that the basophile cells are normally regulated by the neural hypophysis.

The apparent affinity of the neurohypophysis for *l*-thyroxine would suggest that it is part of the pituitary-thyroid axis.

Experiments are in progress to determine if selective localization occurs in other animals.

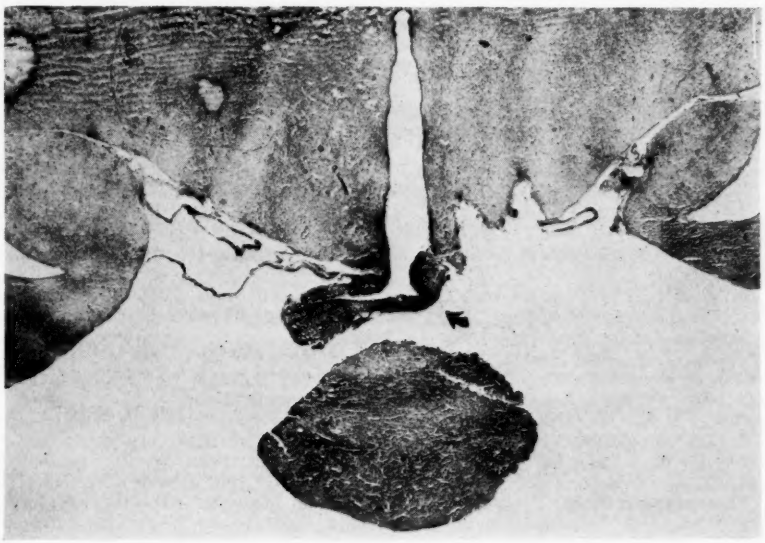

Fig. 5.—Flotation type radioautograph showing localization of radioactive *l*-thyroxine in the infundibulum (arrow).

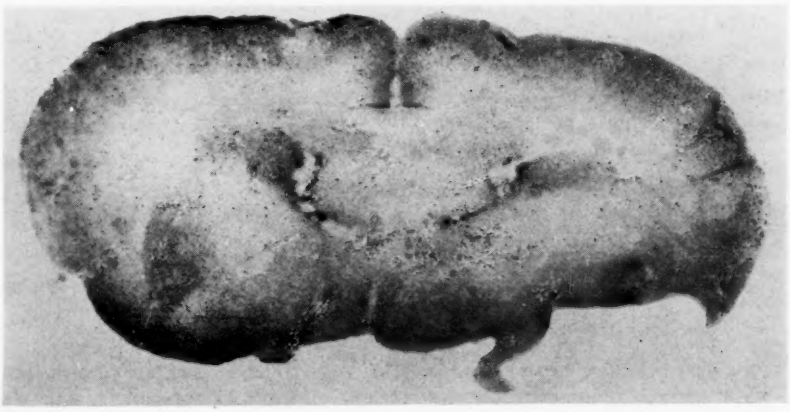

Fig. 6.—Contact radioautograph of section from region of mamillary bodies revealing a diffuse minimal concentration of radioactive *l*-thyroxine.

SUMMARY

Radioactive *l*-thyroxine is shown to selectively localize in the neurohypophysis of rabbits.

The anterior pituitary and brain shows a minimal concentration of radioactive *l*-thyroxine.

Radioactive iodine I^{131} shows no localization in the neurohypophysis, anterior pituitary, or brain.

The possible significance of this observation in controlling thyroid function is briefly discussed.

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DIABETOGENIC ACTION OF ORGANIC REAGENTS: DESTRUCTIVE LESIONS OF ISLETS OF LANGERHANS CAUSED BY SODIUM DIETHYLDITHIOCARBAMATE AND POTASSIUM ETHYLXANTHATE

ICHIRO KADOTA, M.D., AND OSAMU MIDORIKAWA, M.M.
KYOTO, JAPAN

THE production of diabetes with alloxan, by destroying the beta cells in the islets of Langerhans, has evoked a considerable interest in regard to the specific affinity of some chemical structures to the islet cells. However, there have remained as yet many obscure points in the essential mechanism of diabetogenic action. In their studies on the diabetogenic action of alloxan homologues and related compounds, Brückmann and Wertheimer[1] reported no diabetogenic effect of rhodizonic acid—an organic reagent. Patterson[2] has recently demonstrated the diabetogenic effect of dehydroascorbic and dehydroisoascoribe acids, which are similar to alloxan in chemical structure and properties. More recently, he[3] has found a similar effect from dehydroglucoascorbic acid.

As previously reported,[4] it was confirmed that two organic reagents—oxine and dithizone—could cause diabetes by destroying beta cells of the islet of Langerhans. Diabetogenic action of these reagents has been inferred from the histochemical detection of zinc in islet cells,[5] and the close correlation between zinc and insulin.[6] However, the two reagents above are able to react with many metals other than zinc. Furthermore, nickel, cobalt, and cadmium can be used instead of zinc in the crystallization of insulin.[6] It is, therefore, expected that there may be some similar effective agents which may injure islet cells.

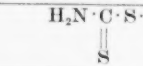
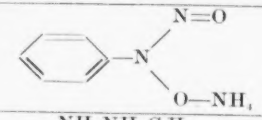
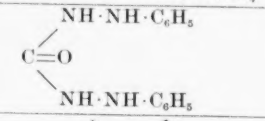
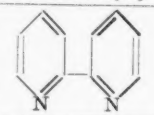
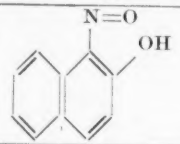
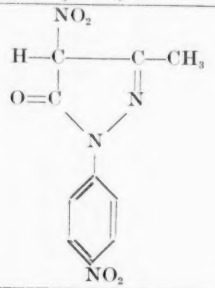
From these considerations present studies have been undertaken on the diabetogenic action of following organic reagents and related compounds. It may be of importance to investigate the chemical specificity of the diabetogenic substances in explaining their destructive mechanism of islet cells.

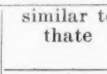
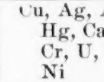
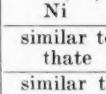
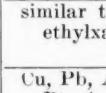
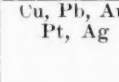
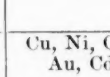
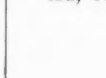
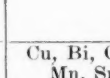
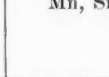
METHODS AND MATERIALS

Organic reagents and related compounds used in this experiment are shown in Table I. For experimental animals adult rabbits were used. Water soluble substances were injected intravenously as aqueous solution. Some insoluble agents were injected intravenously, using proper solvents as indicated in Table I. Blood sugars were determined by Hagedorn-Jensen's method,[7] and urine sugar by Benedict's qualitative method. In case of autopsy, the pancreas was first removed and fixed in Bouin's fluid as soon as possible. For staining the granules in the islet cells, Gomori's chromium-hematoxylin phloxine method[8] was employed. Other tissues were fixed in formalin and examined by routine hematoxylin-eosin staining in necessary cases.

From the Department of Pathology, Kyoto University School of Medicine.
Received for publication, June 12, 1951.

TABLE I. ORGANIC REAGENTS AND RELATED COMPOUNDS USED IN THIS EXPERIMENT AND OUTLINE OF RESULTS

ORGANIC REAGENTS	STRUCTURAL FORMULA	METALS WHICH REACT WITH REAGENTS	SOLUTION FOR INJECTION	INJECTED DOSES	INFLUENCE UPON BLOOD SUGAR
Ammonium dithiocarbamate		Cu, Bi, Pb, Sn, Sb, Ag, Ni, Co, Mn, Al, Zn, Fe	aqueous solution	100-200 mg./kg.	occasionally slight hyperglycemia
Ammonium nitrosophenylhydroxylamine (Cupferron)*		Cu, Fe, Zr, Ti, V, Nb, Ta, Cr, Sn, U, Ga, Bi, Hg, Be, Th	aqueous solution	100 mg./kg.	slight hyperglycemia
Diphenylcarbazide*		Cr, Cd, (Co, Cu, Fe, Pb, Mg, Hg, Ni, Ag, Zn)	saturated solution in aq. ammoniac	7 c.c./kg.	slight hyperglycemia
α,α'-Dipyridyl*		Fe	aqueous solution	100-150 mg./kg.	temporary hyperglycemia
α-Nitroso-β-naphthol*		Co	saturated solution in water or 0.1 aq. ammoniac (warmed)	10 c.c./kg.	no change
Picrolonic acid		Ca, Pb, Cu, K, Mg	saturated aqueous solution	ca. 50 mg./kg.	slight hyperglycemia

Potassium methylxanthate		similar to ethylxanthate	aqueous solution	100-150 mg./kg.	slight hyperglycemia
Potassium ethylxanthate (Potassium ethyldithiocarbamate, Potassium ethylxanthogenate)		Cu, Ag, Au, Zn, Cd, Hg, Ca, Sn, Pb, V, Cr, U, Mn, Fe, Co, Ni	aqueous solution	200-500 mg./kg.	hyperglycemia, occasionally followed by hypoglycemia
Potassium propylxanthate		similar to ethylxanthate	aqueous solution	300-400 mg./kg.	temporary hyperglycemia
Potassium butylxanthate		similar to ethylxanthate	aqueous solution	150 mg./kg.	slight hyperglycemia
Rhodanine		Cu, Pb, Au, Hg, Pd, Pt, Ag	aqueous solution	100-180 mg./kg.	slight hyperglycemia
Salicylaldoxime		Cu, Ni, Co, Mo, Pd, Au, Cd, Pb	solution in diluted alcohol	50-70 mg./kg.	no change
Sodium diethyldithiocarbamate*		Cu, Bi, Cd, Fe, Pb, Mn, Sn, Zn, Mg	aqueous solution	500-1,000 mg./kg.	hyperglycemia occasionally followed by hypoglycemia
Sodium salt of 1-nitroso-2-hydroxynaphthalene-3,6-disulfonate (Nitroso-R-salt)		Co, (Ca, Ba, Fe, Pb, Ni, Ag)	aqueous solution	200-500 mg./kg.	slight hyperglycemia followed by toxic death
Thioglycolic acid (Mercaptoacetic acid)*		Fe	aqueous solution	250 mg./kg.	no change

Data of properties of above organic reagents are taken from Kuroda,[9] Mellah[10] and van Nieuwenburg.[11] Data of properties of xanthates are taken from Atsuki and Takada.[16] Data of salts of fatty acids are taken from Beilsteins *Handbuch der Organischen Chemie*, vierte Auflage, Bd. 2, Berlin, 1920, Julius Springer.

*Commercial products.

TABLE I—CONT'D

ORGANIC REAGENTS	STRUCTURAL FORMULA	METALS WHICH REACT WITH REAGENTS	SOLUTION FOR INJECTION	INJECTED DOSES	INFLUENCE UPON BLOOD SUGAR
Thiosemicarbazide*	$NH_2 \cdot NH—C(=S)—NH_2$	Cu, Os, Ru, Ag	aqueous solution	5-500 mg./kg.	sudden convulsion with hyperglycemia
d-glucose-thio-semicarbazone	$CH_2OH(CHOH)_4CH{=}N \cdot NH \cdot CS \cdot NH_2$		aqueous solution	50-200 mg./kg.	no change
Thiourea*	$NH_2 \cdot CS \cdot NH_2$	Bi, Sb, Cr, Co, Fe, Hg, Ni, Os, Pd, Ru, Ag, Se, Te	aqueous solution	100-300 mg./kg.	slight hyperglycemia
Caproic acid	$C_5H_{11} \cdot COOH$	Na, Cu, Ag, Sr, Ca, Ba, Zn, Pb	neutralized solution with 1N-NaOH	100 mg./kg.	no change
Caprylic acid	$C_7H_{15} \cdot COOH$	Na, Cu, Ag, Ba, Zn, Pb	neutralized solution with 1N-NaOH	50-200 mg./kg.	no change
Capric acid	$C_9H_{19} \cdot COOH$	Na, Cu, Ag, Mg, Ca, Ba	neutralized solution with 1N-NaOH	50-200 mg./kg.	occasionally slight hyperglycemia
Lauric acid	$C_{11}H_{23} \cdot COOH$	Na, K, Li, Mg, Cu, Ag, Ca, Ba, Sr, Zn, Pb, Mn, Co, No	neutralized solution with 1N-NaOH	150 mg./kg.	no change
Undecylic acid	$C_{10}H_{21} \cdot COOH$	Ag, Ba	neutralized solution with 1N-NaOH	150 mg./kg.	no change
Suberic acid	$HOOC(CH_2)_6COOH$	Na, K, Cu, Ag, Mg, Ca, Sr, Ba, Zn, Cd, Hg, Pb, Mn, Co, Ni	neutralized solution with 1N-NaOH	100 mg./kg.	no change

RESULTS

Outline of results with each reagent are presented in Table I.

Changes of Blood Sugar, Caused by Sodium Diethyldithiocarbamate.—Changes of blood sugar in fifteen rabbits after intravenous injection of 500 to 1,000 mg. per kilogram sodium diethyldithiocarbamate are shown in Table II and Fig. 1.

Immediately after injection, all rabbits showed hyperglycemia. Eleven rabbits showed only temporary hyperglycemia, which reached its peak after two hours, and then gradually returned to normal value after twenty-four to forty-eight hours.

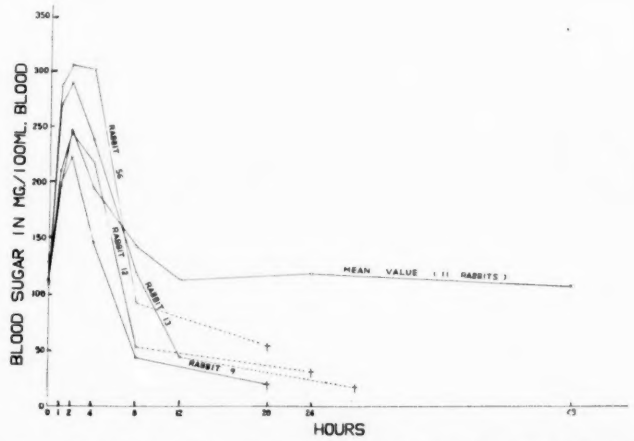


Fig. 1.—Changes of blood sugars forty-eight hours after intravenous injection of 500 to 1,000 mg. per kilogram sodium diethyldithiocarbamate in rabbits.

In four rabbits, after initial hyperglycemia, the blood sugar decreased below normal in eight to twelve hours after injection, and hypoglycemic convulsions were observed. Two of them were found dead after twenty and twenty-four hours. The other two rabbits continued to show hypoglycemia, in spite of repeated administration of glucose, whenever they showed hypoglycemic convulsions. Finally they died of weakness at twenty-eight and thirty hours after the beginning of the injection.

Histologic Changes of Islets of Langerhans in Rabbits That Died From Hypoglycemia, as Caused by Sodium Diethyldithiocarbamate.—In Rabbit 24, which was sacrificed fourteen hours after injection, capillary lesions and widening of the pericapillary spaces were observed. The cytoplasm of some alpha cells were reddish granular stained, with their nuclei almost normally preserved. A few transparent cells, due to depletion of their granules, were located at the periphery of the islets. The islets were chiefly composed of beta cells, with apparently normal granules.

In Rabbit 9, which died from hypoglycemia twenty hours after injection, capillary lesions and disarrangement of islet cells were observed. The nuclei of the majority of the alpha cells were pyknotic, being homogenously deeply

TABLE II. CHANGES OF BLOOD SUGAR AFTER INTRAVENOUS INJECTION OF SODIUM DIETHYLDITHIOCARBAMATE IN RABBITS

RABBIT	BODY WEIGHT (GM.)	INJECTED DOSES (MG./KG.)	BLOOD SUGAR IN MG./100 ML. BLOOD								NOTE
			BEFORE INJECTION	1 HR.	2 HR.	4 HR.	8 HR.	12 HR.	24 HR.	48 HR.	
9	2,200	500	128	198	218	146	43	–	20(after death)		Died after 20 hr.
10	2,450	500	123	278	312	193	184	–	152	101	
11	2,000	500	108	178	221	171	123	–	111	98	
12	2,300	700	110	210	244	218	53	(convulsion)			Died after 30 hr.
13	2,500	700	107	269	289	238	118	44	(convulsion)		Died after 28 hr.
15	1,825	600	128	–	256	258	239	–	176	108	
16	1,850	500	111	187	294	283	115	–	99	109	
17	2,050	700	111	178	226	196	172	139	138	–	
18	1,800	700	103	191	209	163	113	83	100	–	
19	1,710	700	108	226	282	147	100	101	107	–	
20	2,570	900	108	179	195	145	128	128	107	102	
22	2,040	1,000	94	168	169	141	95	–	107	–	
24	1,330	700	101	158	–	–	–	340 (14 hr.)			Sacrificed after 14 hr.
55	1,900	750	127	229	305	257	108	–	88	129	
56	2,150	1,000	113	287	305	302	92	(convulsion)			Died after 24 hr.

TABLE III. CHANGES OF BLOOD SUGAR AFTER INTRAVENOUS INJECTION OF POTASSIUM ETHYLXANTHATE IN RABBITS

RABBIT	BODY WEIGHT (GM.)	INJECTED DOSES (MG./KG.)	BLOOD SUGAR IN MG./100 ML. BLOOD									NOTE
			BEFORE INJECTION	1 HR.	2 HR.	4 HR.	8 HR.	12 HR	24 HR.	48 HR.	72 HR.	
34	2,400	200	126	223	298	342	251	138	47	36 (40 hr.)		Died after 40 hr.
35	1,870	200	113	–	–	308	–	274	211	138	114	
37	1,700	200	107	–	228	247	–	76	41	(convulsion)		Died after 55 hr.
38	1,730	200	91	–	255	283	–	–	147	122	101	
40	1,970	250	102	–	177	244	–	–	106	122	97	
47	1,890	250	107	–	337	247	–	217	144	165	156	
48	1,770	220	118	–	258	160	–	136	263	342	374	
51	2,000	250	102	–	98	342	–	196	141	112		
63	2,000	300	112	–	272	283	184	–	100	153		
65	2,260	350	109	208	179	147	98	–	66	(convulsion)		Died after 42 hr.
66	2,250	200	105	199	234	227	206	–	173			
82	2,100	200	117	–	244	150	63	–	188	140	117	
83	2,400	200	111	243	311	264	–	–	134	117	129	
84	1,800	200	110	174	264	249	260	–	212	102	99	
85	1,850	250	100	148	264	356	265	–	231	178	130	

TABLE IV. CHANGES OF BLOOD SUGAR AFTER INTRAVENOUS INJECTION OF LARGE DOSES POTASSIUM ETHYLXANTHATE DIVIDED OVER INTERVALS IN RABBITS

RABBIT	BODY WEIGHT (GM.)	INJECTED DOSES (MG./KG.)	MODE OF INJECTION	BLOOD SUGAR IN MG./100 ML. BLOOD					NOTE
				BEFORE INJECTION	1 HR.	2 HR.	4 HR.	14 HR.	
74	2,500	500	200 mg./kg. after 20 minutes	119	356 (after death)				Died after 35 minutes
75	2,550	450	3 times in 50 minutes	103	313	424	458 (after death)		Died after 4 hr. 35 min.
76	2,100	450	100 mg./kg. after 40 minutes	147	264	248	212	66 (after death)	Died after 14 hr.
79	2,530	350	100 mg./kg. after 30 minutes	127	263	288	302		Died after 18 hr. from hypoglycemia
81	2,250	300	one time	132	186	161	143		Convulsions after 12 hr., died after 23 hr.

TABLE V. CHANGES OF BLOOD SUGAR AFTER LNTRAVENOUS INJECTION OF POTASSIUM METHYLXANTHATE, POTASSIUM PROPYLXANTHATE AND POTASSIUM BUTYLXANTHATE IN RABBITS

RABBIT	BODY WEIGHT (GM.)	REAGENT	INJECTED DOSES (MG./KG.)	BLOOD SUGAR IN MG./100 ML. BLOOD						
				BEFORE INJECTION	1 HR.	2 HR.	4 HR.	12 HR.	24 HR.	48 HR.
58	2,050	Methylxanthate	100	122	104	118	123	–	85	–
60	1,940	Methylxanthate	150	156	182	190	208	–	106	–
67	2,320	Propylxanthate	300	90	141	109	180	–	138	–
70	2,030	Propylxanthate	300	100	267	258	214	–	139	–
73	2,340	Propylxanthate	400	105	–	304	211	143	127	136
63	2,000	Propylxanthate	250	140	–	345	–	–	179	109
53	2,100	Propylxanthate	200	126	–	298	–	–	–	–
71	1,960	Butylxanthate	150	105	135	127	126	–	–	–

stained. Their cytoplasm, however, appeared almost normal except for pale staining in some cells. Some nuclei of the beta cells showed pyknosis exceptionally, but most nuclei and granules of their cytoplasm were almost normally preserved, with exceptional diminution of the granules (Fig. 2). Some islets were composed of beta cells, surrounded by a few scattered degenerated alpha cells at the periphery of the islet (Fig. 3).

In Rabbit 56, which died after twenty-four hours, capillary lesions were noted. While nuclei of alpha cells appeared normal, their cytoplasm showed evidence of degeneration, staining pale, and appearing granular reddish. The beta cells were generally swollen, their nuclear membranes were prominent, and their cytoplasm appeared quite transparent as a result of depletion of the granules (Fig. 4).

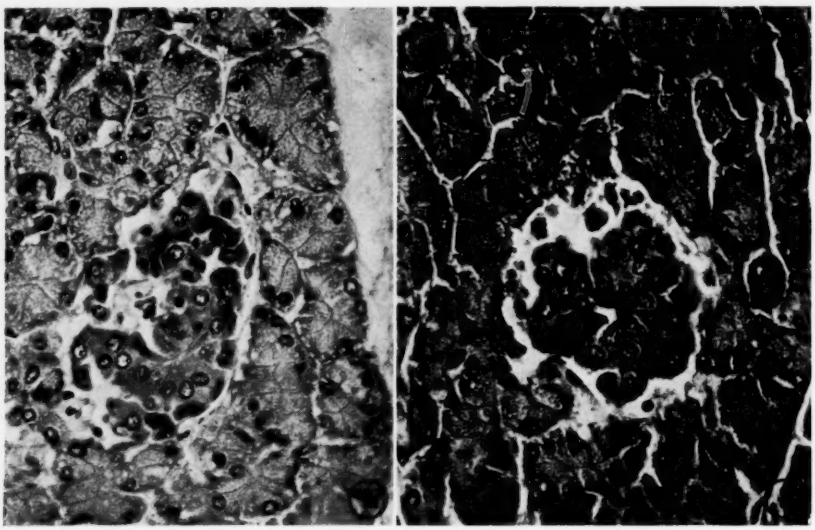


Fig. 2. Fig. 3.

Fig. 2.—Islet of Langerhans of Rabbit 9, which died from hypoglycemia about twenty hours after intravenous injection of sodium diethyldithiocarbamate. All nuclei of alpha cells appear pyknotic, whereas beta cells are apparently normal. (Gomori's chromium-hematoxylin phloxine stain.)

Fig. 3.—Islet of Langerhans of Rabbit 9, which died from hypoglycemia about twenty hours after intravenous injection of sodium diethyldithiocarbamate. While centers of islets are composed of normal appearing beta cells, alpha cells show severe signs of degeneration, such as pyknosis of nuclei and disintegration of cytoplasm, at periphery of islet. (Gomori's chromium-hematoxylin phloxine stain.)

In Rabbit 13, which continued hypoglycemic for fourteen to eighteen hours after injection, widening of the spaces between cell cords was observed in the islets. There were almost no alpha cells, which stain reddish with phloxine. Although the nuclei of the beta cells remained almost normal, their cytoplasm appeared transparent and were stained pale violet, leaving some granules at one part of some cells.

In Rabbit 12, which continued hypoglycemic for eight to thirty hours after injection, lesions of the islets were the most conspicuous. Islets were generally

Fig. 4.

Fig. 5.

Fig. 4.—Islet of Langerhans of Rabbit 56, which died from hypoglycemia about twenty-four hours after intravenous injection of sodium diethyldithiocarbamate. Periphery of islet is stained granular reddish due to necrosis of alpha cells. Most of beta cells show transparent cytoplasm with prominent nuclei. Their boundary is not clear. (Gomori's chromium-hematoxylin phloxine stain.)

Fig. 5.—Islet of Langerhans of Rabbit 12, which continued hypoglycemic from eight to thirty hours after intravenous injection of sodium diethyldithiocarbamate. Islet is swollen and composed of transparent and hydropic cells which are presumably beta cells. There is no normal showing of alpha cells. Only a few parts of islet are stained reddish with phloxine, showing remaining cytoplasm of alpha cells. (Gomori's chromium-hematoxylin phloxine stain.)

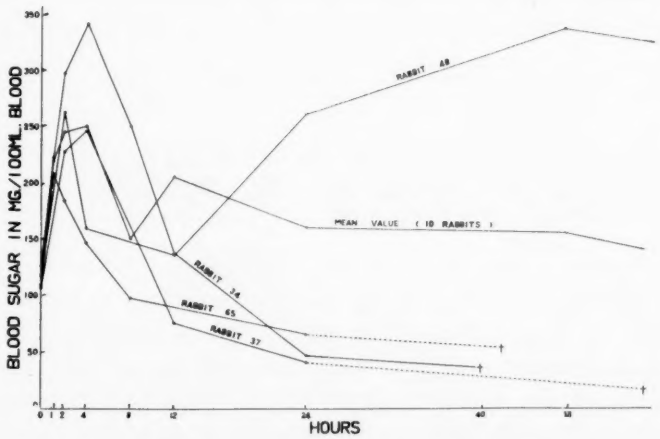


Fig. 6.—Changes of blood sugars during forty-eight hours after intravenous injection of 200 to 500 mg. per kilogram potassium ethylxanthate in rabbits.

swollen and composed of large swollen transparent cells. Only one part of the periphery of the islets was slightly stained reddish with phloxine. There remained almost no alpha cells, showing normal appearance. The composing cells, which might be regarded as beta cells, were generally swollen, with large prominent nuclei and cytoplasm showing a transparent, hydropic and netlike structure, as a sequel of complete loss of granules (Figs. 5 and 7).

Changes of Blood Sugar Caused by Potassium Ethylxanthate.—Changes of blood sugar in fifteen rabbits after intravenous injection of 200 to 350 mg. per kilogram are shown in Table III and Fig. 6. In each rabbit the blood sugar increased immediately after injection, reached its peaks two to four

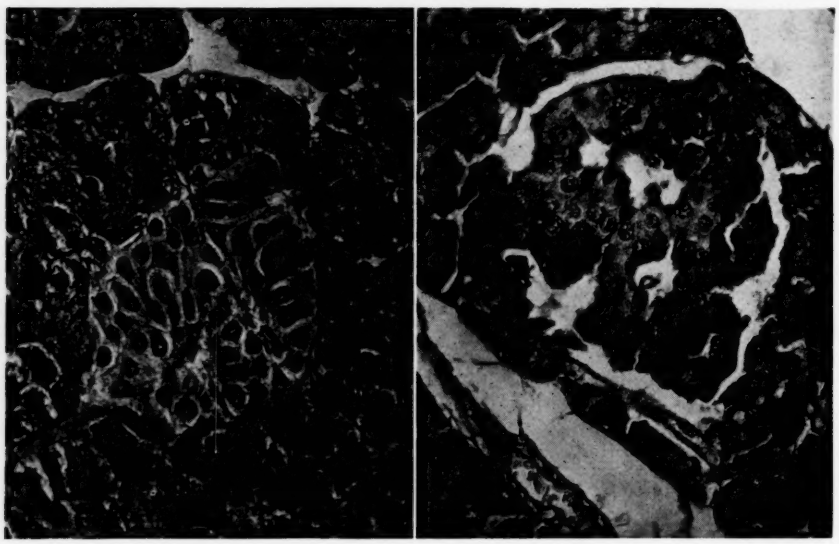


Fig. 7. Fig. 8.

Fig. 7.—Islet of Langerhans of Rabbit 12, which continued hypoglycemic from eight to thirty hours after intravenous injection of sodium diethyldithiocarbamate. Islet is composed of large hydropic cells. Their cytoplasm appears hydropic and shows netlike structure. There remains no reddish-stained alpha cells. (Gomori's chromium-hematoxylin phloxine stain.)

Fig. 8.—Islet of Langerhans of Rabbit 79, which died from hypoglycemia eighteen hours after intravenous injection of potassium ethylxanthate. Spaces between cords of islet cells are widened, due to capillary lesions. Most of beta cells are stained pale due to exhaustion of their granules. Alpha cells are well preserved except for slightly degenerated appearance. (Gomori's chromium-hematoxylin phloxine stain.)

hours after injection, and then decreased gradually. In eleven rabbits, the blood sugar gradually returned to normal after twenty-four hours. However, three rabbits showed hypoglycemia after eight to twelve hours, accompanied by hypoglycemic convulsions. Whenever they showed hypoglycemic convulsion, 5 to 10 Gm. of glucose were administered intravenously. In spite of repeated injection of glucose, all rabbits died from forty to fifty-five hours after injection.

In only one rabbit (Rabbit 48), blood sugar increased after twenty-four hours followed by permanent hyperglycemia. While the same doses of potas-

sium ethylxanthate were injected after two weeks, this rabbit continued hyperglycemic (200 to 300 mg. per cent), showing some fluctuation during two months.

As animals could not tolerate single injection of doses over 350 mg. per kilogram of potassium ethylxanthate, 300 to 500 mg. per kilogram of potassium ethylxanthate were administered intravenously, divided in small doses over short intervals. Changes of blood sugar in these cases are shown in Table IV.

Two rabbits, which were administered 450 to 500 mg. per kilogram, divided over a twenty to fifty minutes' interval, showed sudden marked hyperglycemia, followed by death. Three rabbits, given 300 to 450 mg. per kilogram injection, showed hyperglycemia one to four hours after injection, followed by hypoglycemia after twelve to eighteen hours. Two rabbits were found dead after fourteen and eighteen hours, respectively, and the other rabbit died after twenty-three hours, despite repeated administration of glucose.

Of two rabbits given daily intravenous injection of potassium ethylxanthate in doses of 200 mg. per kilogram, one died after four days and the other died after twenty days (in doses of 100 mg. per kilogram in the last seven days) showing no marked changes of blood sugar.

Changes of Blood Sugar Caused by Other Xanthates.—The outline of results is as shown in Table V. Slight temporary hyperglycemia was observed in each case. Propylxanthate showed considerable effect, but methylxanthate and butylxanthate appeared to be somewhat toxic and less effective.

Histologic Changes of Islet Caused by Administration of Potassium Ethylxanthate.—In Rabbit 74, which died after thirty-five minutes from marked hyperglycemia, capillary exudation due to lesions of their endothelium and subsequently widening of spaces between cords of islet cells were observed. However, alpha cells and beta cells appeared normal.

In Rabbit 75, which died after four hours and thirty-five minutes, the islet cells showed marked disarrangement. Although alpha cells appeared almost normal, except for signs of slight degeneration in some cells, beta cells showed pyknosis of nuclei and pale staining cytoplasm accompanied by some manifestation of degeneration.

In Rabbits 76 and 79, which died from a single hypoglycemic attack, after fourteen and eighteen hours, respectively, the islet cells were separated from one another, due to capillary lesions. Nuclei of alpha cells were partly pyknotic, and their cytoplasms were stained reddish, appearing granular or cloudy as a result of degeneration. The nuclei of beta cells were pyknotic, partly showing distinct nuclear membranes. Their cytoplasm appeared transparent, due to complete depletion of their granules, leaving a few granules in one part of the islet (Fig. 8). In addition to the above findings, some beta cells showed conspicuous necrosis and disintegration.

In Rabbit 81, which continued hypoglycemic for twelve to twenty-three hours, slight degeneration of alpha cells, and degranulation and transparency of beta cells were observed. The changes in the beta cells in this case were quite similar to those in the rabbit showing hypoglycemia after injection of

sodium diethyldithiocarbamate (Fig. 9). In Rabbit 65, which continued hypoglycemic for twenty-four to forty-two hours, pyknosis of nuclei and degranulation and disintegration of cytoplasm of the beta cells were conspicuous, with a slight degeneration of the alpha cells.

In Rabbit 37, which continued hypoglycemic for twenty-four to fifty-five hours after injection, the most remarkable histologic alterations of the islets were observed. While alpha cells appeared almost normal, the centers of the islets were replaced extensively by pale violet stained necrotic, homogenous masses, in which a few shadows of necrotic nuclei were seen scattered (Fig. 10). In other, relatively slightly damaged islets, necrotic findings of beta cells were distinctly observed. In some islets alpha cells showed disarrangement, with signs of degeneration (Fig. 11).

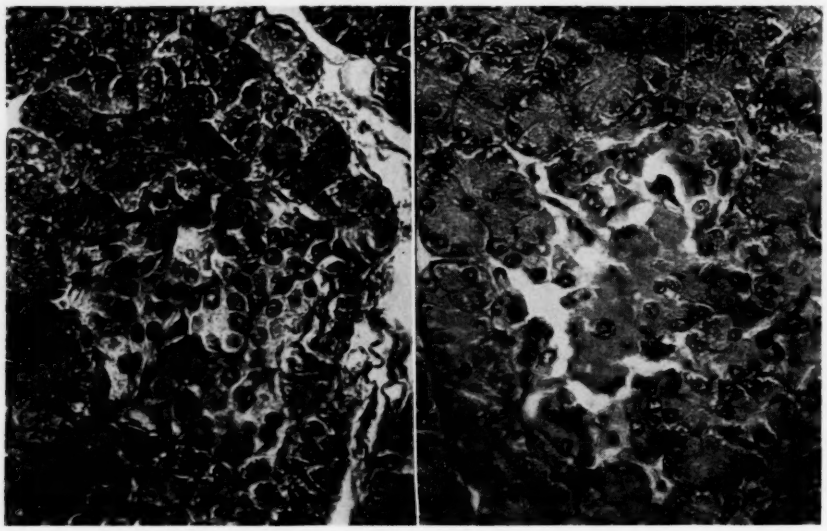


Fig. 9. Fig. 10.

Fig. 9.—Islet of Langerhans of Rabbit 81, which continued hypoglycemic from twelve to twenty-three hours after intravenous injection of potassium ethylxanthate. Most alpha cells show degenerated appearances, such as pyknosis of nuclei and disintegration of cytoplasm. Cytoplasm of beta cells are hydropic, with complete loss of granules. (Gomori's chromium-hematoxylin phloxine stain.)

Fig. 10.—Islet of Langerhans of Rabbit 37, which continued hypoglycemic from twenty-four to fifty-five hours after intravenous injection of potassium ethylxanthate. While alpha cells are well preserved at the periphery, the center of islet is replaced by pale, violet stained necrotic mass. (Gomori's chromium-hematoxylin phloxine stain.)

In rabbits given daily intravenous injection of potassium ethylxanthate, disarrangement of islet cells and widening of spaces between cords of islet cells, owing to exudation from capillaries, were observed. While alpha cells were well preserved, beta cells were generally reduced in number, showing pyknotic nuclei or large nuclei which might be considered as signs of degeneration of beta cells. And their cytoplasm appeared clear with some loss of granules (Fig. 12).

Characteristic Changes Caused by Thiosemicarbazide.—After thirty minutes following intravenous injection of 500 mg. per kilogram thiosemicarbazide, the

rabbit showed sudden convulsion followed by death. Terminal blood sugar was 319 mg. per cent (before injection it was 95 mg. per cent).

Another animal given 50 mg. per kilogram thiosemicarbazide injection showed similar sudden convulsions thirty-eight minutes after the injection. Although this animal was able to recover temporarily with 20 units of insulin, it died after one hour and six minutes with a second convulsion (terminal blood sugar was 195 mg. per cent).

Fig. 11.

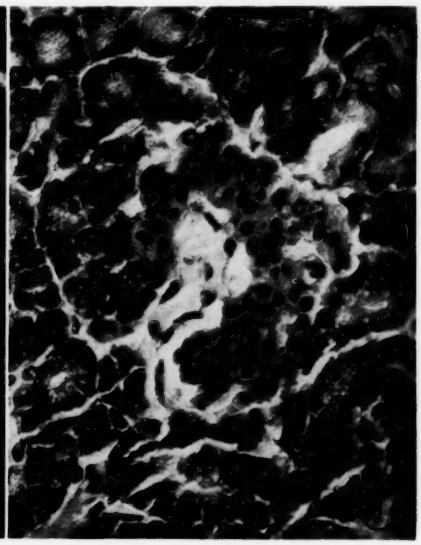


Fig. 12.

Fig. 11.—Islet of Langerhans of Rabbit 37, which continued hypoglycemic from twenty-four to fifty-five hours after intravenous injection of potassium ethylxanthate. In center of islet, necrotic findings of beta cells are conspicuous. Most of beta cells show degenerated appearance. Alpha cells are arranged irregularly with considerable degeneration. (Gomori's chromium-hematoxylin phloxine stain.)

Fig. 12.—Islet of Langerhans of Rabbit 83, which was given daily intravenous injection of potassium ethylxanthate during twenty days. Islet shows marked capillary lesions and exudation from capillary. Most beta cells show marked pyknotic nuclei, while alpha cells appear normal. (Gomori's chromium-hematoxylin phloxine stain.)

The third rabbit, administered 5 mg. per kilogram of thiosemicarbazide, showed convulsions after two hours and twenty-five minutes. This animal apparently recovered completely after 20 units of insulin. Subsequently, it showed four times similar convulsions at approximately one-hour intervals. In each case of convulsions insulin was administered subcutaneously in total doses of 230 units. However, it finally died after seven hours and ten minutes. In this case, islets showed no marked changes except for slight diminution of beta granules and capillary lesions with widening of pericapillary spaces.

Following intravenous administration of 50 to 200 mg. per kilogram d-glucose-thiosemicarbazone, animals showed no changes of blood sugar and no occurrence of convulsion as observed in thiosemicarbazide.

Changes of Blood Sugar by Other Organic Compounds.—The outline of influences on blood sugar of organic compounds other than above-mentioned

agents is shown in Table I. Increase of blood sugar, returning to normal values approximately twenty-four hours after injection, was shown as temporary hyperglycemia. Slight increase of blood sugar (about 150 mg. per cent) after one to four hours was shown as slight hyperglycemia.

Various fatty acids caused no remarkable changes on blood sugar except for capric acid. In some cases capric acid caused temporary hyperglycemia without visible effects in most animals.

DISCUSSION

Since the discovery of alloxan diabetes, studies on diabetogenic action have been performed with substances related to alloxan in their chemical structures, and with those which show similarity in chemical properties. Among substances related to alloxan, the diabetogenic action of N-methyl-, ethyl-, and propyl alloxan, alloxantin, dimethylalloxan, diethylalloxan, dialuric, and methyldialuric acids has been confirmed.[1] Thereafter, similar effect of dehydrosascorbic acid, dehydroisoascorbic acid,[2] and dehydroglucoascorbic acid[3] has been reported.

However, no diabetogenic substance has as yet been discovered which has similar chemical properties with alloxan, since little is known about the mechanism by which alloxan exerts damaging effect on islet cells. As stated, the diabetogenic action of oxine and dithizone[4] was observed on the basis of another idea. Since these two reagents show many common properties in reaction with metals, it may be presumed that other organic reagents may have a similar effect on islet cells. As described, the following characteristic substances were found to be effective.

Sodium diethyldithiocarbamate[9, 10, 11] is a white, crystalline powder, soluble in water, which reacts and forms precipitates with Cu, Bi, Cd, Fe Pb, Mn, Sn, Zn, and Mg, etc. Its reactions with Cu (1:100,000,000) and Zn (1:20,000,000) are especially sensitive.[11] This compound was found to be able to cause hyperglycemia and frequently subsequent hypoglycemia in experimental animals. Histologic alterations in the islets caused by this agent were very characteristic. In cases with initial hypoglycemia, degeneration of alpha cells was conspicuous with capillary lesions. In animals which continued hypoglycemic for long periods, the cytoplasm of alpha cells disappeared almost completely, and the islets were filled with large transparent cells with depletion of granules. These findings in the islets are quite different from those in the hypoglycemic states, as produced by alloxan, oxine, and dithizone. No remarkable necrosis or disintegration was observed in beta cells of the islets.

These histologic findings of the islets indicate the continuous process of degeneration and disappearance of alpha cells. From these histologic alterations it may be presumed that irrecoverable hypoglycemia can be attributed to important disturbances due to lack of alpha cell function. This hypoglycemia is quite different from that caused by alloxan, from which animals recovered after only one or two administrations of glucose.

Thorogood and Zimmermann[12] found that removal of the pancreas caused ketosis and coma rapidly in alloxan diabetic dogs, when administration of insulin was withheld. These changes may be attributed to lack of alpha cell function and may be considered as a marked disturbance comparable to irrecoverable hypoglycemia with destruction of alpha cells caused by sodium diethyldithiocarbamate.

From observations[13, 14, 15] that alpha cells contain the hyperglycemic factor, it may be explained that initial hyperglycemia may have some possible correlation to alpha cells. Subsequent hypoglycemia may be interpreted as being referable to hyperfunction of beta cells or liberation of insulin.

In short, irrecoverable hypoglycemia was caused by sodium diethyldithiocarbamate. But histologic alterations in islets consisted in destruction of alpha cells and hydropic appearance of remaining cells.

Alkali xanthates[16] are yellowish-white crystalline materials, having a characteristic, unpleasant odor. Their solubilities in water decrease with the increase of the molecular weight of alcohols. The xanthates of various metallic elements are formed from the interaction between alkali xanthates and metals. The color of xanthates is characterized by their salts. Therefore, potassium ethylxanthate has been used as an analytical reagent.[16, 17] As described, potassium ethylxanthate was found to be able to cause marked changes of blood sugar and destructive lesions in islet cells. Potassium propylxanthate was found to produce transitory hyperglycemia, whereas potassium methylxanthate and potassium butylxanthate appeared to be less effective.

Histologic alterations of islets induced by potassium ethylxanthate indicated the direct action of the reagent on islets early after injection. Though necrosis and disintegration of beta cells were observed in the most severe hypoglycemic cases, degenerative lesions of beta cells were generally less than those caused by alloxan, oxine, and dithizone. However, it was found that the were always accompanied by degenerative findings of alpha cells. According to some investigators,[18] some alpha cells may suffer greater injury leading to necrosis of single cells together with disintegration of beta cells following injection of alloxan. Furthermore, some cases are reported in which all of the cells of both alpha and beta types are apparently destroyed.[18] In further examinations of necrotic islets of hypoglycemic rabbits caused by dithizone, degenerative changes of alpha cells such as pyknosis of nuclei were occasionally observed.

These facts indicate that islet-damaging substances may exert their effect not only on beta cells, but also, even slightly, on alpha cells.

In some cases lesions of alpha cells of islets caused by potassium ethylxanthate closely resembled those caused by sodium diethyldithiocarbamate. Irrecoverable hypoglycemia by potassium ethylxanthate may also be attributed to lesions of islets accompanied by degeneration of alpha cells, as in cases of sodium diethyldithiocarbamate.

Therefore, it may also be expected that animals will show diabetes if they recover from hypoglycemia, considering the necrotic findings of beta cells. No

effects or slight effects of other xanthates may be attributed to the differences in chemical properties according to alcohol radicals.

Absence of any effect of ammonium dithiocarbamate, which is similar to sodium diethyldithiocarbamate and potassium ethylxanthate in molecular formula and properties, may be referable to its easily decomposable properties.

Thiosemicarbazide[10] forms complex compounds with Cu, Os, Ru, and Ag as organic reagent. It is used for isolation and identification of aldehyde and ketone.[19] This agent could cause characteristic convulsion with hyperglycemia, even with low doses. But it is difficult to explain why insulin can relieve animals, even temporarily, from convulsions caused by thiosemicarbazide. In order to make its action clear, d-glucose-thiosemicarbazone—one of thiosemicarbazone compounds—was tested in animals without effect. Therefore, characteristic changes may be attributed to some specific chemical action of thiosemicarbazide itself. However, it may not be due simply to hyperglycemia because the grade of hyperglycemia is not always so severe. Further studies will be necessary to clarify the mechanism of described action of thiosemicarbazide.

It is well known that fatty acids react with metal ions such as Ca, Mg, Zn, and Cu, forming their metal soaps. But their abilities as organic reagents are not so sensitive. For instance, sensitivity of α-amino-n-caproic acid in reaction with Zn is described as 1:5,000.[11] Their cytotoxic effects are different according to their chemical properties. Among tested fatty acids, capric acid, which has injurious effect on cells, caused occasionally temporary hyperglycemia. Furthermore, other organic reagents showed tendencies to elevate blood sugar temporarily without remarkable changes.

As indicated, sensitive organic reagents having cytotoxic effect may have possibility to cause destructive lesions of islets. Their injurious effect on islet cells may be attributed to direct action, due to their specific affinity to the islet cells. Each agent will show differences in affinity to the two types of islet cells, according to their chemical properties.

Further studies on correlation between chemical specificity and lesions of islet cells may disclose the damaging mechanism of diabetogenic substances.

SUMMARY

Diabetogenic action of twenty-four organic reagents and related compounds was examined in rabbits.

Following intravenous injection of 500 to 1,000 mg. per kilogram sodium diethyldithiocarbamate, all rabbits showed hyperglycemia immediately. Subsequently, four rabbits of fifteen showed severe hypoglycemia after eight to twelve hours, from which they did not recover after administration of glucose.

In initial hyperglycemia induced by sodium diethyldithiocarbamate, islets showed slight degeneration of alpha cells. In the subsequent hypoglycemia, degenerative lesions of alpha cells were observed with slight degeneration of beta cells. In persisting hypoglycemia, continuing for twenty-eight to thirty hours after injection, disappearance of alpha cells and complete degranulation of beta cells were confirmed.

After intravenous injection of 200 to 300 mg. per kilogram potassium ethylxanthate in fifteen rabbits, all rabbits showed hyperglycemia. Subsequently, three of them showed hypoglycemia twelve to twenty-four hours after injection, and died after forty to fifty-five hours, in spite of repeated injections of glucose. One showed permanent hyperglycemia which continued for two months after initial hyperglycemia. After intravenous injection of 300 to 500 mg. per kilogram potassium ethylxanthate divided at intervals, all rabbits died from severe initial hyperglycemia, or from subsequent hypoglycemia.

In initial hyperglycemia caused by potassium ethylxanthate, islets showed capillary lesions, slight degeneration of alpha cells, and degeneration of beta cells. In the early hypoglycemic phase, remarkable degeneration and transparent appearance of beta cells, due to depletion of granules in the cytoplasm, were observed, with slight lesions of the alpha cells.

In hypoglycemia for long periods, the islets showed necrosis and disintegration of beta cells. In severe cases, the centers of the islets turned to homogenous necrotic masses, surrounded by alpha cells. After repeated injection of potassium ethylxanthate, the islets showed decreased beta cells with their degenerative findings such as pyknosis of nuclei.

After intravenous injection of 5 to 500 mg. per kilogram thiosemicarbazide, animals showed sudden convulsions, with hyperglycemia after two hours to twenty minutes. Although these animals survived temporarily by administration of insulin, they always died later. Islets of these animals appeared almost normal.

Some of the chemicals used in this experiment have been observed to cause temporary hyperglycemia.

We are greatly indebted to Dr. H. Horii for his invaluable suggestions and generous supply of many reagents throughout this study. The photomicrographs were made by Mr. R. Tada.

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IMPAIRMENT OF LIVER FUNCTION SUBSEQUENT TO INJURY TO THE SPINAL CORD

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IN A PREVIOUS study of the metabolic effects of injury to the spinal cord, attention was called to the impairment of liver function of three patients which occurred within two weeks after they had been injured.[1] Thompson and Rice have observed hepatomegaly in thirty-three of 250 paraplegic patients under their care, but these authors did not report any abnormality of the liver function of their patients.[2] Hepatomegaly also has been observed to follow subtotal removal of the spinal cord of a human being.[3] Beecher,[4] in a study of severely wounded soldiers, found retention of bromsulfalein in fifty-nine cases to be 12.4 ± 1.2 per cent within the first twenty-four hours after injury. He did not find any correlation between the degree of retention of bromsulfalein and shock, nor could retention be correlated with the amount of blood lost. Beecher[4] did not indicate whether his series of wounded soldiers included cases of injury to the spinal cord.

The present study deals with the effect of acute severe injury to the spinal cord on the liver function of human beings.

MATERIAL AND METHODS

Several tests for liver function and clinical and laboratory observations were carried out on eight consecutive patients, each of whom had suffered an injury to the spinal cord severe enough to cause paraplegia. No patient was included in this study who came under our observation longer than seven days after incurrence of the injury. In each case the significant tests were repeated on at least three different days, and, in all instances, when abnormal results were obtained, the tests were repeated periodically at least once weekly for ten weeks.

The laboratory studies which were carried out, and the normal value for each were: (1) bromsulfalein or BSP liver function test (less than 10 per cent retention sixty minutes after injection of the dye); (2) cephalin-cholesterol flocculation test (flocculation not greater than 1, on grading basis of 1 to 4 with 1 being the lowest grade and 4 the highest); (3) determinations for serum bilirubin (direct: negative, indirect: less than 0.7 mg. per 100 ml. serum); (4) determination for cholesterol (150 to 250 mg. per 100 ml. plasma); (5) determinations for cholesterol esters (100 to 175 mg. per 100 ml. plasma), and (6) determination for blood urea (10 to 40 mg. per 100 ml. of whole blood). In addition, the post-traumatic catabolic reaction in six of the eight cases was evaluated by daily determinations of the serum proteins (6.4 to 7.8 Gm. per 100 ml. serum), the albumin-globulin ratio (1.5:1 to 3:1), and the urinary excretion of nitrogen, creatine, and pre-formed creatinine. Although the diet

From the Mayo Foundation, and the Mayo Clinic.
Received for publication, July 16, 1951.

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of the patients was not rigidly controlled, accurate measurements of protein and caloric intake were maintained, and the daily nitrogen balance of the latter six patients was determined.

RESULTS

Significant and marked degrees of impairment were indicated by the bromsulfalein test for liver function in six of the eight cases in our series. The retention of bromsulfalein sixty minutes after injection was 40 per cent (grade 4) in three cases and 38 per cent, 28 per cent, and 26 per cent (grade 3), respectively, in the remaining three cases. Two of the eight patients failed to demonstrate any retention of dye thirty minutes after injection of bromsulfalein. In each instance the impairment demonstrated by the bromsulfalein test became apparent within ten days after injury to the spinal cord. In one case the bromsulfalein test indicated impairment, grade 3 (26 per cent retention) as early as the third day after injury. None of the patients was jaundiced at any time during a ten-week period of observation. Moreover, the degree of retention of bromsulfalein was not related to loss of blood (absent or negligible in six of the eight cases), elevation of temperature (tests were performed only when the patients were afebrile), or transfusions of blood. It did not appear to be related to the level of injury to the spinal cord, cervical, thoracic, and lumbar lesions being represented in this small series. In five of the six cases in which the results of this test (BSP) were abnormal, gradual spontaneous return to normal limits occurred within ten weeks after injury. In one instance, impairment, grade 4, as indicated by the BSP test, still was present at the time of the patient's death ten weeks after injury. The results of the bromsulfalein test for liver function are correlated with other significant data from these eight cases in Table I.

TABLE I. CORRELATION OF RESULTS OF BROMSULFALEIN TEST FOR LIVER FUNCTION OF 8 PATIENTS, AFTER INJURY TO THE SPINAL CORD, WITH OTHER LABORATORY AND CLINICAL DATA

CASES	AGE AND SEX	HIGHEST DEGREE OF BSP RETENTION AFTER SIXTY MINUTES (PER CENT)	TIME THAT RESULTS OF BSP TEST REMAINED ABNORMAL (WEEKS)	BILIRUBIN (HIGHEST VALUES RECORDED, MG. PER 100 ML. SERUM)		LEVEL OF SPINAL CORD TRANSECTION	HIGHEST CONCENTRATION OF UREA (MG. PER 100 ML. BLOOD)
				DIRECT	INDIRECT		
1	46 M	40	9	0.7	1.1	C-7	90
2	44 M	0	–	0	0	T-12	46
3	68 M	38	5	0.7	0.8	C-5	94
4	68 M	40	Still abnormal at 10 weeks	0	1.2	C-6	54
5	26 M	26	10	0	1.4	T-4	32
6	18 F	40	6	2.1	1.7	T-11	24
7	12 M	0	–	0	1.2	T-1	30
8	45 M	28	3	0.9	0.6	L-1	48

The results of the cephalin-cholesterol flocculation test were abnormal in Case 3 only. There was slight elevation of either the direct or indirect bilirubin determinations in seven cases, but the elevation was quantitatively significant in Case 3 only, and the serum cholesterol and cholesterol esters were abnormally low in the same case. The concentration of urea was elevated

to more than 40 mg. per 100 ml. of blood in five cases. This elevation occurred during the first week after injury, and returned to normal by the third week after injury. The elevation of blood urea in these cases was interpreted as a reflection of the post-traumatic catabolism of body protein. No patient in this series showed evidence of renal insufficiency.

Each patient showed evidence of advanced post-traumatic catabolism of body protein as reflected in abnormally high excretions of nitrogen and creatine in the urine, a fall in serum proteins, and a negative nitrogen balance. The catabolic reaction was extremely severe in the first four cases, the average negative nitrogen balance being −14 Gm. daily for the first fourteen days after injury. The average nitrogen balance in the other four cases was −4 Gm. of nitrogen daily for the first fourteen days after injury. In the latter four cases the patients received testosterone propionate in an attempt to lessen the post-traumatic catabolism of body protein.[5] Further specific data concerning studies of nitrogen balance and the use of testosterone as an anticatabolic agent are recorded elsewhere and are not germane to this report.[6] It is considered worthy of note, however, that a quantitative relationship did not appear to exist between the retention of bromsulfalein and the degree of the catabolic reaction after injury to the spinal cord.

COMMENT

The results of our investigation cannot be readily interpreted, and both the pathogenesis of the impairment of liver function and its relation, if any, to other metabolic abnormalities observed among paraplegic patients must await further elucidation. Gynecomastia has been observed to occur among a considerable number of paraplegic men,[1, 7, 8] but a relationship between gynecomastia and impairment of liver function of men who have suffered injuries to the spinal cord never has been established. It would appear that the results of the bromsulfalein retention test are specifically affected in these cases, but the significance of this fact is not clear. The use of additional tests for liver function in such cases may aid in clarifying the pathologic physiology underlying the effect of injury to the spinal cord on liver function.

The mechanism by which injury to the spinal cord may result in impairment of the bromsulfalein test also is uncertain. The average degree of retention of dye of the eight patients in this series was 27.7 per cent (this average includes the two patients who did not demonstrate retention of the dye). This degree of dye retention is more than twice as great as that observed by Beecher[4] for severely wounded soldiers, and indicates that some mechanism of the spinal cord is probably involved in production of the abnormal response. Selye has reported cloudy swelling and a decrease in the size of the liver during the alarm reaction. He attributed the latter to the decrease in blood volume and protein catbolism which follows acute stress.[9] He stated that in some cases these changes will progress to result in extensive atrophy, focal necrosis and fatty infiltration of the liver.[10] Although injury to the spinal cord has been demonstrated to result in the alarm reaction,[11] protein catabolism[1] and vasodilatation below the level of the lesion of the spinal cord "throughout

the entire arterial system from the root of the aorta to the capillaries,[12] the role which these factors may play in producing impairment of liver function or pathologic changes in the liver has not yet been determined. Considerably more factual information must be obtained before a sound hypothesis can be offered as to the cause of impairment of liver function after injury to the spinal cord.

SUMMARY

In six of eight consecutive cases of acute severe injury to the spinal cord, impairment of liver function was evidenced by the bromsulfalein test for liver function. The abnormal results of the bromsulfalein test did not appear to be related to elevation of temperature, loss of blood, the severity of the post-traumatic catabolic reaction, or to the level of the lesion of the spinal cord. In the six cases which demonstrated abnormality of liver function, the impairment, according to the bromsulfalein test, appeared within ten days after injury to the spinal cord, and resolved spontaneously within ten weeks after injury in five of these six cases.

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THE IMPORTANCE OF SERIAL DETERMINATIONS OF SERUM ALKALINE PHOSPHATASE IN INCOMPLETE BILIARY TRACT OBSTRUCTION

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SINCE Roberts[1] observed increased concentrations of alkaline phosphatase in the sera of patients with obstructive jaundice, the value of this determination in the differential diagnosis of jaundice has been established. Although a number of limitations have been indicated, there appears to be general agreement that elevations of serum alkaline phosphatase concentrations above 10 Bodansky units, in patients with jaundice, usually indicate biliary obstruction. On the other hand, high concentrations may be found inexplicably in certain nonobstructive conditions, but elevations of this nature are inconstant and variable.[2-7] In the bulk of instances icterus has been a concomitant of increase in serum alkaline phosphatase activity, but it has been noted that in certain cases with incomplete biliary obstruction there has not been parallel elevation of serum bilirubin. The precise mechanism of this dissociation has not been established, and experimental studies[8-15] have indicated that several factors may play roles. Among these are the degree of obstruction and the amount of hepatic damage. Our own investigations in dogs[15] suggest that the intensity of cholangitis and intrahepatic abscess formation are additional factors which may contribute to this paradox.

Among our cases with biliary tract obstruction we have encountered a small group of patients with incomplete biliary obstruction whose sera have exhibited an interesting sequence of alterations. Early in the course of the disease, these individuals have shown a marked elevation of serum bilirubin and only a slight increase in the alkaline phosphatase activity. Later, as the bilirubin levels declined, significant increases in phosphatase occurred. We have, also, observed patients in whom the alkaline phosphatase concentrations remained elevated after removal of an obstructing lesion, although the serum bilirubin returned to normal.

The present report cites clinical data in which the phenomenon of dissociation of phosphatase and bilirubin was encountered. We believe these observations emphasize the value of serial determinations of alkaline phosphatase in revealing the presence of partial biliary obstruction. To this end we have selected as examples four cases with incomplete biliary tract obstruction. In

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Read, in part, before the Twenty-third Annual Meeting of the Central Society for Clinical Research, Chicago, Ill., Nov. 4, 1950.

Aided by grants from the Robert Gould Research Foundation, The Milton Brown Memorial Fund, and the John Eisman Memorial Fund.

Received for publication, June 22, 1951.

every instance, liver biopsy was obtained before, during, or after operation. Also included are the data obtained from a dog from an incomplete experimental series of studies on the effects of biliary obstruction on liver function tests.[15] The findings in this animal are considered of contributory interest, since identical serial blood studies were also carried out.

The serum alkaline phosphatase activity was determined by the Bodansky method[16] and the serum bilirubin concentration by the method of Ducci and Watson.[17] Other liver function tests performed included the thymol turbidity,[18] zinc sulfate turbidity,[19] cephalin cholesterol flocculation,[20] total serum lipids,[21] total and esterified serum cholesterol,[22] and the partition of serum proteins.[23]

CASE STUDIES

CASE 1.—*Cholelithiasis With Partial Occlusion of the Common Duct by an Inflammatory Granuloma:* M. M., a 68-year-old white woman, was known to have had pernicious anemia for which she had received vitamin B_{12} during a six-month period preceding her entry into the hospital. Four weeks before admission she noted the onset of moderately severe, intermittent pain in the right upper abdomen accompanied by anorexia, but without nausea, vomiting, or chills. Dark urine and light stools appeared three days, and jaundice one day prior to admission.

During a hospital admission eight months earlier, liver function studies were normal except for a 3 plus cephalin flocculation test. A liver biopsy performed at that time showed a moderate degree of fatty vacuolization without other histologic abnormality.

Physical Findings: There was a moderate degree of icterus. The temperature was 99.2° F. The liver extended 4 cm. below the right costal margin, was firm, and moderately tender. The gall bladder and spleen were not palpable.

Liver function studies are shown in Table I.

TABLE I. LIVER FUNCTION STUDIES (CASE 1, M. M.)

Hospital day	2	7	12	17	21	23	31	38
Thymol turbidity (1-4 units)	11	10	8	9		9	7	6
Zinc sulfate turbidity (5-12 units)	17	22	20	23		22	21	18
Cephalin flocculation (0-2 plus/24 hours)	3	3	4	4		4	3	3
Total serum lipids (475-725 mg. %)	715	850	950	800		750	650	635
Serum bilirubin								
Prompt (0.2 mg. %)	8.0	2.15	2.0	3.5		1.0	0.6	0.4
Total (1.2 mg. %)	11.0	4.5	4.5	6.0		2.4	1.2	1.2
Serum alkaline phosphatase (1-4 B.U. %)*	5	27	21	23		11	4	3
Serum cholesterol								
Total (150-260 mg. %)			275					184
Ester (112-187 mg. %)			193					116
Ester % (70-75%)			70					63
Prothrombin concentration (100%)		71						
Serum proteins								
Total (6.70-7.20 Gm. %)			7.3					6.02
Albumin (3.60-3.85 Gm. %)			3.35					4.0
Globulin (3.10-3.35 Gm. %)			3.95					2.02
Bromsulfalein retention (less than 5%)†								10

SURGERY

Figures in parentheses indicate normal values.

*Bodansky units.

†Five milligrams per kilogram body weight at forty-five minutes.

Course: Repeated turbidity and cephalin flocculation tests showed no appreciable change from the admission values. Determinations of total serum bilirubin and serum alkaline phosphatase are depicted in Table I. On admission, the serum bilirubin was moderately elevated and the alkaline phosphatase was just above normal. Five days later, there was a significant rise in the alkaline phosphatase, but a fall in the serum bilirubin. On the twenty-first day cholecystectomy was performed. Operation included the removal of a cystic duct stone and a nonspecific inflammatory granuloma which was found to be partially surrounding and constricting the common bile duct at a point 4 cm. distal to the juncture of the main hepatic ducts. Thereafter, both the bilirubin and phosphatase returned to normal levels, the latter lagging somewhat behind the former.

A needle specimen of the liver procured thirteen days before operation showed bile stasis and a moderately severe cholangitis involving the interlobular biliary ductules. The latter exhibited active proliferation and considerable infiltration with neutrophiles and lymphocytes.

CASE 2.—*Cholelithiasis With Common Duct Stone:* G. G., a 67-year-old white man, noted the onset of intermittent, cramping pain in the right upper abdomen five days before admission. Anorexia, nausea, vomiting, and mild chills were present. Dark urine and jaundice were noted three days before admission. Since the age of 50 years the patient had been aware of fatty food intolerance. He had been a moderate alcoholic and had been admitted to mental hospitals on several occasions because of paranoid behavior.

Examination revealed the patient to be acutely ill and mildly icteric. The temperature was 101° F. There were no vascular spiders, and no palmar erythema was present. The liver edge was felt 2 cm. below the right costal margin and was slightly tender. The gall bladder and spleen were not palpable.

The results of serial liver function tests representative of many performed at various times during the patient's illness are shown in Table II.

Course: The temperature ranged between 99° F. and 102° F. for six weeks despite antibiotic therapy. During this period the blood urea nitrogen rose to 90 mg. per cent. The liver function studies showed evidence of severe hepatic dysfunction early in the course, with a gradual improvement as the disease became more chronic. The patient became paranoid and refused surgery. As his condition again deteriorated, he finally consented to surgical exploration. On the one hundred thirty-second hospital day, a common duct stone was removed. Following operation, the serum bilirubin returned to normal but the alkaline phosphatase remained elevated.

Three liver specimens were removed over a five-month period. At the initial study on the seventh hospital day, the section revealed an acute suppurative cholangitis of moderately severe degree. A second needle specimen on the forty-third day showed the cholangitis to be less intense but associated with an increased amount of nonspecific intralobular degenerative alteration. The last specimen procured at the time of operation on the one hundred thirty-second day revealed a residuum of the early inflammatory process with some increase in the amount of portal area fibrous tissue and bile duct reduplication. Bile stasis in the intralobular canaliculi continued throughout the illness.

CASE 3.—*Carcinoma of the Cystic Duct With Partial Occlusion of the Common Duct:* H. C., a 75-year-old white man, a known alcoholic, had had intermittent swelling of the abdomen and edema of the legs over a two-year period. Two weeks prior to admission, the abdominal swelling became marked and was accompanied by dark, reddish-brown urine and light colored stools. For 2 months, he had had marked anorexia and nausea, but no vomiting. Despite the presence of edema, marked weight loss had been noted. There had been recurrent bouts of diffuse, cramping abdominal pain, chilly sensations, fever and sweats.

Examination revealed a chronically ill patient with a mild degree of icterus of the skin and sclerae. The temperature was 98° F. The abdomen was moderately distended with ascites. The liver was irregular, firm, nontender, and extended 6 cm. below the right costal margin. The gall bladder and splen could not be palpated. There was 3 plus edema of both lower extremities.

Serial liver function studies are recorded in Table III.

TABLE II. LIVER FUNCTION STUDIES (CASE 2, C. G.)

Hospital day	2	16	28	40	54	88	118	130	132	144	152	158
Thymol turbidity (1-4 units)	7	23	14	14	15	14	19	17		9	6	6
Zinc sulfate turbidity (5-12 units)	15	36	26	31	28	32	39	35		27	32	32
Cephalin flocculation (0-2 plus/24 hours)	4	4	4	4	4	4	4	4		4	4	4
Total serum lipids (475-725 mg. %)	560	610	620	690	640	800	1130	830		660	680	650
Serum bilirubin												
Prompt (0.2 mg. %)	8.0	22.0	4.5	3.0	5.2	1.2	2.2	1.2	S	1.0	0.7	0.6
Total (1.2 mg. %)	12.0	36.0	8.0	5.5	9.0	2.0	4.0	2.0	U	2.3	1.3	1.2
Serum alkaline phosphatase (1-4 B.U. %)*	5.2	8.0	15	18	51	40	24	15	R	12	15	14
Serum cholesterol									G			
Total (150-260 mg. %)		150		129		208			E		195	
Ester (112-187 mg. %)		0		63		131			R		128	
Ester % (70-75%)		0		49		63			Y		65	
Prothrombin concentration (100%)	75	38	53		54	80		72		100		
Serum proteins		6.35										
Total (6.70-7.20 Gm. %)		2.15		7.65			5.5				6.16	
Albumin (3.60-3.85 Gm. %)		4.20		2.50			2.0				2.80	
Globulin (3.10-3.35 Gm. %)				5.15			3.5				3.36	
Bromsulfalein retention (less than 5%)†						32						18

Figures in parentheses indicate normal values.

*Bodansky units.

†Five milligrams per kilogram body weight at 45 minutes.

TABLE III. LIVER FUNCTION STUDIES (CASE 3, H. C.)

Hospital day	3	10	18	34	115	128	140	142	152	170	190	200	226
Thymol turbidity (1-4 units)	4	7	7	10	19	4		3	4	6	5	4	3
Zinc sulfate turbidity (5-12 units)	12	17	28	21	16	9		8	12	14	12	12	11
Cephalin flocculation (0-2 plus/24 hours)	3	0	0	2	3	0		0	2	3	0	0	0
Total serum lipids (475-725 mg. %)	1085	870	775	915	1180	535		580	550	525	620	620	525
Serum bilirubin													
Prompt (0.2 mg. %)	2.7	2.6	2.5	2.5	5.0	8.7	S	3.5	2.0	1.25	0.5	0.3	0.1
Total (1.2 mg. %)	3.0	5.0	6.0	3.5	8.0	15.0	U	6.0	4.5	2.6	1.0	0.9	0.5
Serum alkaline phosphatase (1-4 B.U. %)*	34	43	38	37	47	54	R G E R Y	31	53	49	53	38	20
Serum cholesterol													
Total (150-260 mg. %)			313			200							176
Ester (112-187 mg. %)			186			57							69
Ester % (70-75 %)			59			29							39
Prothrombin concentration (100%)	100	100			55	49			78			66	78
Serum proteins													
Total (6.70-7.20 Gm. %)	5.3		6.95		5.45	5.15			5.05		5.5		5.5
Albumin (3.60-3.85 Gm. %)	2.75		2.35		1.7	1.9			2.0		2.7		2.0
Globulin (3.10-3.35 Gm. %)	2.55		4.6		3.75	3.25			3.05		2.8		3.5
Bromsulfalein retention (less than 5%)†												65	

Figures in parentheses indicate normal values.

*Bodansky units.

†Five milligrams per kilogram body weight at 45 minutes.

Course: The ascites and edema responded to a low salt diet, ammonium chloride, and mercurial diuretics as evidenced by diuresis and a twenty-five-pound weight loss in a nine-day period. Beginning on the fourth hospital day and recurring during the remainder of the preoperative course, he had several febrile periods with the temperature ranging between 99° F. and 102° F. The liver function tests showed increased abnormalities in the turbidity tests, while the cephalin flocculation was positive on occasions.

Needle biopsy specimens obtained on the thirteenth and thirty-fourth hospital days revealed identical alterations. There was universal enlargement of portal areas which were the seat of fibrosis and active bile duct proliferation. Periductal inflammation of acute and chronic nature was a prominent feature. The lesion was identified as an early stage of biliary cirrhosis.

The patient refused operation and left the hospital, only to return seventy-nine days later in an extremely poor state of nutrition. Examination showed an increase in icterus and the presence of marked ascites and edema. Supportive therapy was given in preparation for surgery. Laparotomy, one hundred thirty-eight days after the initial entry into the hospital, revaled a carcinoma of the cystic duct with partial encroachment on the common duct but without evidence of metastasis. The tumor was removed and an end-to-end anastomosis of the common bile duct was made.

After operation, the serum bilirubin gradually returned to normal, but the serum phosphatase remained elevated until the fifty-second postoperative day, after which there was a steady decrease. During this period, there was a rapid accumulation of ascites. Three abdominal paracenteses were performed, and on each occasion the ascitic fluid showed an alkaline phosphatase activity of greater than 3 Bodansky units per 100 milliliters. The presence of the enzyme in the ascitic fluid may partially explain its falling concentration in the serum.

CASE 4.—*Cholelithiasis With Common Duct Stones:* E. J., a 68-year-old man, had been well until three weeks before admission to the hospital, at which time he noted the onset of severe epigastric pain which radiated to the back. There was associated nausea and vomiting, but he had had no chills. A pale stool was noted three days before admission. There was no previous history suggestive of liver or gall bladder disease.

On examination the patient appeared acutely ill. There was moderate icterus of the skin and sclerae. The temperature was 100° F. Abdominal findings consisted of tenderness in the epigastrium and right upper abdomen.

Course: Shortly after admission the patient's temperature rose to 102° F. and reached 104° F. on the third hospital day. A blood culture showed the presence of *Aerobacter aerogenes.* The fever persisted despite the administration of penicillin and streptomycin. Liver function tests are recorded in Table IV.

Because of the patient's poor physical condition, operation was delayed until the twelfth hospital day when a cholecystectomy and choledochoduodenostomy were performed with removal of stones from the gall bladder and common bile duct. The postoperative course was complicated by the development of a subdiaphragmatic abscess, and the patient was not discharged from the hospital until the eightieth day. Following operation, the serum bilirubin returned to normal, but the alkalne phosphatase remained elevated.

A year and a half later, the patient was readmitted to the hospital because of weakness and fever. At this time he was found to be emaciated. There was no icterus of the sclerae. The temperature was 101.6° F. The liver was firm, and extended 3 cm. below the right costal margin. The spleen was palpable 2 cm. below the left rib margin. Liver function studies of this admission are also shown in Table IV.

Portal areas as seen in a needle biopsy specimen of liver removed four hundred seventy-five days after surgical release of biliary obstruction were moderately fibrotic and contained multiple biliary ductules no longer in active proliferation. The related inflammatory exudate consisting of lymphocytes and neutrophiles was sparse. The process representative of biliary cirrhosis was undoubtedly of long standing and an inactive state.

EXPERIMENTAL STUDY

The dissociation of serum alakline phosphatase and bilirubin levels observed clinically has been reproduced experimentally in dogs by several investigators, following ligation of the individual hepatic bile ducts draining various portions of the liver.[9, 10] These observers noted a rapid increase in serum phosphatase activity shortly after operation, although jaundice did not develop. The alkaline phosphatase reached a peak concentration between the first and second weeks after operation, and then gradually declined to approximately normal values by the end of two months. The amount of increase in the phosphatase varied with the number of ducts ligated. Post-mortem examination of the obstructed liver lobes showed only slight histologic changes.

In studying the alterations in liver function tests in experimental obstructive jaundice in dogs,[15] we have produced incomplete biliary tract obstruction by ligation of the left main hepatic bile duct which results in the interruption of bile flow from the gall bladder and approximately two-thirds of the liver. The data obtained from one such animal are representative of a group of five animals, and are shown in Fig. 1 and Table V. After ligation of the left main bile duct, there was a rapid increase in the alkaline phosphatase concentration, with only a slight rise in serum bilirubin. The latter returned to the control value on the twenty-eighth day and remained at that level throughout the experimental period. The peak concentration of alkaline phosphatase activity was reached on the twenty-fifth day. A rapid decline was noted up to the forty-second day, and then a more gradual decrease until the one hundred twenty-eighth day. In the final thirty days of the experiment, the serum phosphatase remained at a rather constant level of 15 units.

The animal was sacrificed on the one hundred sixty-second day. Gross examination of the obstructed portions of the biliary tract showed a thickened, contracted, fibrotic gall bladder, stenotic bile ducts, and an increased consistency of the liver. The unobstructed bile ducts and liver lobes appeared normal. Post-mortem sections of the obstructed lobes revealed frank active obstructive biliary cirrhosis. Perilobular ductules showed advanced and continuing proliferation, and there was an associated exudate of marked degree consisting of intermixed lymphocytes and neutrophiles. Within the lobules several granulomatous abscesses were encountered. These contained liberated bile in considerable amounts. A major branch of the portal vein was thrombosed and organized. Sections from the nonobstructed lobes demonstrated minor nonspecific inflammatory and degenerative lesions unrelated to the ductular tree.

DISCUSSION

The case examples cited have demonstrated the dissociation of the serum bilirubin and alkaline phosphatase values which may occur in the course of incomplete biliary tract obstruction. The serum bilirubin may be markedly increased early in the course of the disease. This is possibly attributable to hepatitis related to hydrohepatosis and ascending infection. At this stage serum alkaline phosphatase is normal or only slightly elevated, resulting perhaps

TABLE IV. LIVER FUNCTION STUDIES (CASE 4, E. J.)

Hospital day	2	4	10	12	21	40	80		1	5
Thymol turbidity (1-4 units)	3	3	7		10	8	8		7	9
Zinc sulfate turbidity (5-12 units)									20	21
Cephalin flocculation (0-2 plus/24 hours)	0	2	3		3	4	4		4	4
Total serum lipids (475-725 mg. %)								555	720	710
Serum bilirubin				S				D		
Prompt (0.2 mg. %)	8.0	18.0	15.0	U	1.6	0.7	0.3	A	0.5	0.4
Total (1.2 mg. %)	10.0	28.0	24.0	R	3.0	1.5	1.3	Y	1.7	1.6
Serum alkaline phosphatase (1-4 B.U. %)*	9	9	20	G	10	9	7		15	14
Serum cholesterol				E				L		
Total (150-260 mg. %)							171			213
Ester (112-187 mg. %)				R			108	A		127
Ester % (70-75%)				Y			63	P		59
Prothrombin concentration (100%)	90	50	81			85		S	53	
Serum proteins								E		
Total (6.70-7.20 Gm. %)										7.45
Albumin (3.60-3.85 Gm. %)										3.70
Globulin (3.10-3.35 Gm. %)										3.75
Bromsulfalein retention (less than 5 %)†							4			16

Figures in parentheses indicate normal values.

*Bodansky units.

†Five milligrams per kilo body weight at 45 minutes.

TABLE V. LIVER FUNCTION STUDIES (DOG NO. 763)

Postoperative day	5	12	20	25	28	34	42	70	110	128	155
Thymol turbidity (1-2 units)	1	2	2	4	2	2	2	2	2	2	4
Zinc sulfate turbidity (2-4 units)	2	4	6.5	7.5	6.5	7.5	11	12	12.5	13.5	13.5
Total serum lipids (500-600 mg. %)	1190	980	1140	900	785	695	745	590	520	625	650
Serum bilirubin											
Prompt (0.1 mg. %)	4.3	1.0	0.6	0.2	0.2	0.2	0.15	0.1	0.1	0.1	0.1
Total (0.4 mg. %)	6.7	2.0	0.9	0.5	0.3	0.3	0.3	0.2	0.2	0.3	0.2
Serum alkaline phosphatase (1-2 B.U. %)*	18	32	82	90	88	72	38	35	25	16	15
Serum cholesterol											
Total (146 mg. %)	392	450	421	417	346	321	313	221	133	136	150
Ester (92 mg. %)	281	268	278	287	232	208	218	146	79	80	87
Ester % (65-75%)	72	60	66	69	67	65	70	66	59	59	58
Serum proteins											
Total (4.9 Gm. %)	5.3	6.15	5.4	5.75	6.75	7.1	6.55	5.65	4.75	5.75	4.95
Albumin (2.75 Gm. %)	3.05	2.8	2.45	2.45	2.45	2.35	2.15	1.8	1.4	1.35	1.1
Globulin (2.15 Gm. %)	2.25	3.35	2.95	3.3	4.3	4.75	4.4	3.85	3.35	4.4	3.85

Figures in parentheses indicate average control values.
*Bodansky units.
Cephalin Flocculation is normally positive in the dog.

from impaired hepatic function (Tables I and II). With progression of the disease repeated determinations may show a marked rise in the serum alkaline phosphatase with a fall in serum bilirubin concentrations. The period of time in which this may occur varies in different patients. In Case 1, this was noted nine days after the onset of jaundice, whereas in Case 2 it was not until the twenty-seventh day of jaundice that a rise above 10 Bodansky units was observed. Liver biopsies during this phase may show intralobular alterations as well as severe cholangitis.

When the obstruction has been present for a longer period of time and the disease process is less acute, the alkaline phosphatase, having become elevated, remains at a high concentration while the serum bilirubin may be only slightly abnormal. During this phase, fluctuations in the concentrations of serum phosphatase and bilirubin are noted to occur simultaneously. Microscopic examination of liver specimens may reveal acute and chronic cholangitis with early fibrosis.

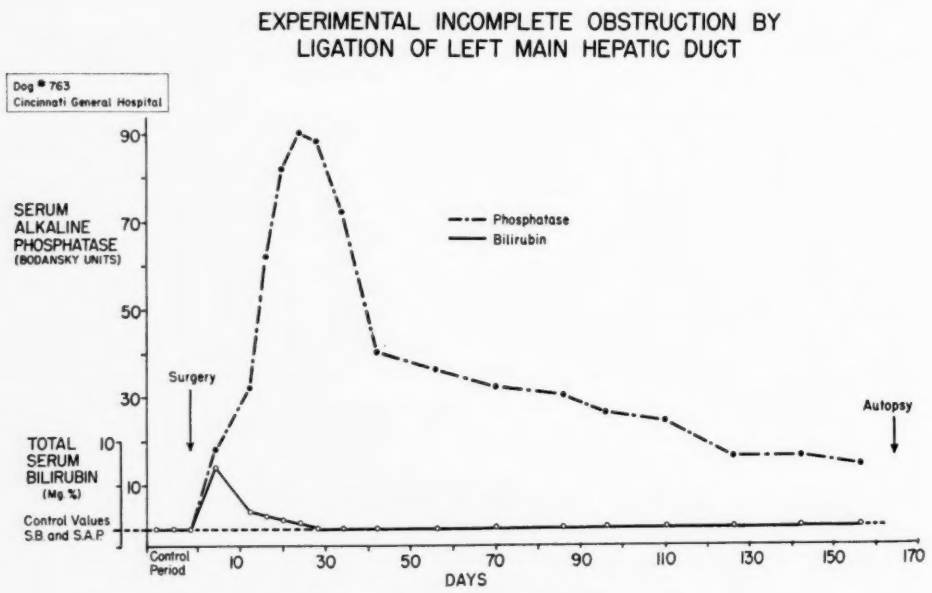

Fig. 1.

After surgical correction of an obstructing lesion, both the serum bilirubin and alkaline phosphatase generally return to normal. However, in some instances serial determinations show a persistent elevation of the alkaline phosphatase without increase of the serum bilirubin. Histologic examination of the liver at this time may reveal the presence of a persistent cholangitis or early biliary cirrhosis.

In experimental obstruction, similar findings were noted in the concentrations of the serial determinations of the serum bilirubin and serum alkaline

phosphatase (Fig. 1). Sections of the liver showed an extensive obstructive cirrhosis with acute and chronic cholangitis.

The recognition of persistent cholangitis with associated early obstructive cirrhosis is of practical importance, since it furnishes an indication for antibiotic therapy and further observation of the patient.

SUMMARY AND CONCLUSIONS

1. This report cites serial studies of the serum bilirubin and serum alkaline phosphatase concentrations in four patients and an experimental animal with partial biliary tract obstruction.

2. It was noted that early in the course of the obstruction serum bilirubin elevation was not necessarily accompanied by a rise in serum alkaline phosphatase. With release or continuation of the obstruction, however, the bilirubin level having been reduced, it was observed that alkaline phosphatase activity increased and often remained elevated for considerable periods of time, even in the absence of jaundice.

3. The changes in the serum alkaline phosphatase and bilirubin concentrations may depend not only on the degree of biliary obstruction, but also on the extent of liver damage secondary to the inflammatory process in the biliary tree, although variations in the production of the two substances may also be of importance. Thus, with increasing liver damage, the serum bilirubin may rise, whereas the serum alkaline phosphatase may remain stationary or decrease. Conversely, with the subsidence of the hepatitis, the serum alkaline phosphatase may rise while the serum bilirubin falls.

4. Biopsy of the liver under these circumstances demonstrated the common denominator of inflammation of the smaller biliary ductules or its sequela, biliary cirrhosis.

5. The serum alkaline phosphatase concentration is a more sensitive indicator of incomplete biliary obstruction than the serum bilirubin, and should be determined repeatedly even after an obstructing lesion has been removed and jaundice has completely subsided.

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THE EFFECT OF SINGLE LARGE DOSES OF CHOLINE OR METHIONINE ON PHOSPHOLIPID TURNOVER IN NORMAL HUMAN SUBJECTS MAINTAINED ON A LOW-PROTEIN DIET FOR SEVEN DAYS*

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THE time required for an inadequate diet to produce fatty infiltration of the liver in the normal human being is not known. Experiences on man indicate that when physiologic stress (anesthesia) is induced, a fatty liver may develop within hours in human subjects who have been eating a normal diet adequate in protective substances.

When a large dose of choline or methionine is given to patients with fatty livers, the turnover of phospholipids is increased.[1] Presumably the patient's previous intake of these protective substances has been inadequate. Once the deficiency has been corrected and the patient resumes an adequate diet, further administration of lipotropic substances does not alter the phospholipid turnover. Normal subjects on an adequate diet have no change in the rate of phospholipid turnover following the administration of lipotropic materials.[2]

Since stress may bring about fatty infiltration of the liver in human subjects on an adequate diet, the same stress occurring in a patient on a borderline or inadequate diet might be expected to induce the same pathologic change in a shorter time, or even to induce a greater alteration. It would be useful, therefore, to know the length of time necessary to bring about early fatty change by the administration of an inadequate diet. If stress, such as an operation or possible exposure to injury, could be anticipated, the administration of protective substances during the interim might prevent damage to the liver, or shorten the period of convalescence from such damage.

The present experiments were undertaken to determine whether the ingestion of an experimental diet low in protein for a short period of time might induce a sufficient alteration in the fat metabolism of normal adults to be detected by the phospholipid turnover. Radioactive phosphorus was used as an indicator.

MATERIAL AND METHOD

Twelve medical students with no history or physical evidence of liver disease were studied. The twelve subjects—eleven males and one female—were divided into two

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This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

The P^{32} used in these experiments was obtained from the National Laboratories, Oak Ridge, Tenn., on allocation from the U. S. Atomic Energy Commission.

Received for publication, June 4, 1951.

*Technical assistance was given by Jesse C. Trott, Jr.

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groups of six each. The two groups were treated in the same fashion, except that group A received choline, group B methionine. In each subject the serum levels of albumin and globulin were determined and various liver function tests were performed. These included bromsulfalein retention, hippuric acid excretion after the injection of benzoic acid, thymol turbidity, and cephalin flocculation. No laboratory evidence of disturbed liver function was found in any of the subjects before or after the experimental diet was given.

Low Protein Diet and a Test Dose of Lipotropic Material.—All subjects were given a carefully controlled diet, containing 32 Gm. of protein (7.6 per cent), 100 Gm. of fat, and 287 Gm. of carbohydrate, and totaling 2,176 calories daily. This diet was continued for nine days.* On the seventh day, all subjects received an intramuscular injection of 0.5 mc. of P^{32} in the form of NaH_2PO_4. One group was then given 10 Gm. of choline chloride and the other 10 Gm. of methionine (dl) by mouth. Blood samples were then taken at 0, 24, and 48 hours following the injection of P^{32}, and the radioactivity and phosphorus content of the lipids and of the inorganic fractions of the plasma were determined.[2]

Low Protein Diet Without a Test Dose of Lipotropic Material.—After six to twelve months on an uncontrolled but adequate diet, the subjects were again maintained for a period of nine days on the same experimental diet. After the seventh day the rate of phospholipid synthesis was restudied with P^{32}, but without the administration of choline or methionine.

Adequate Diet With a Test Dose of Lipotropic Material.—The same subjects were studied a third time after they had been on an adequate diet. Another injection of P^{32} was given, the same lipotropic material was administered, and the radioactivity and phosphorus determinations were repeated.

RESULTS

The specific activity, the relative specific activity, and the newly formed phospholipids have been calculated as a measurement of phospholipid synthesis (Table I). Only one subject (R. I.) maintained on the experimental diet showed a significant increase in phospholipid turnover following the administration of lipotropic material. Three others (K. W., L. C. G., and W. P.) showed a slight increase. Liver function studies repeated at the end of each period of observation were unchanged.

DISCUSSION

Under the conditions of our experiments, an increase in the formation of phospholipids may be assumed when higher values for total radioactivity are found, when the specific activity and relative specific activity values are also higher, and when the newly formed phospholipids are increased. Various factors such as differences in the rate of absorption and in the equilibrium between intra- and extracellular phosphorus may conceivably produce changes in the specific activity of the inorganic phosphorus fraction, but comparisons of the relative specific activity should provide means of reducing these causes of error.

*Four male albino rats, weighing 100 to 110 Gm., were maintained on representative samples of this diet for seven days. On the seventh day each rat was given an intraperitoneal injection of radiophosphorus. Five minutes before the injection the following substances were administered by stomach tube: Rat A—water (3 ml.); rat B—DI-methionine (90 mg. in 3 ml. of water); rats C and D—choline chloride (30 mg. in 1 ml. of water). After six hours the livers were removed and the lipid and inorganic phosphorus were analyzed.[5] The respective relative specific activities observed were as follows: rat A, 67; rat B, 87; rats C and D, 117 and 86. These findings indicate that the administration of a single dose of lipotropic agent stimulated lipid phosphorylation in the liver of the rats maintained on this diet as long as seven days.

The Relation of Phospholipid Turnover to Lipotropic Substances.—The administration of choline to normal individuals on an adequate diet or to cirrhotic patients after therapy with lipotropic substances will not alter the phospholipid turnover. The stimulating effect is detected only when the supply of choline or its precursors has been inadequate and fatty infiltration of the liver is present. Untreated cirrhotic patients with large fatty livers show an increase in plasma phospholipid synthesis after an initial dose of choline or methionine.[3]

TABLE I. PHOSPHOLIPID TURNOVER IN NORMAL INDIVIDUALS WITH AND WITHOUT A SINGLE DOSE OF METHIONINE AND OF CHOLINE AFTER SEVEN DAYS ON A LOW PROTEIN DIET AND AFTER AN ADEQUATE DIET

			24 HOUR			48 HOUR		
STUDENT	MATERIAL GIVEN	DIET	SPECIFIC ACTIVITY	RELATIVE SPECIFIC ACTIVITY	NEWLY FORMED PHOSPHOLIPIDS	SPECIFIC ACTIVITY	RELATIVE SPECIFIC ACTIVITY	NEWLY FORMED PHOSPHOLIPIDS
R. I.	Methionine	low protein	13.3	39.0	1.83	21.1	99.0	4.45
	None	low protein	6.2	22.1	0.78	12.2	58.1	2.48
	Methionine	adequate	9.4	33.5	1.99	15.0	80.2	4.23
W. P.	Methionine	low protein	13.4	42.4	2.24	20.7	87.0	4.28
	None	low protein	5.1	16.1	0.76	19.4	91.2	3.68
	Methionine	adequate	8.9	27.0	1.24	14.2	91.0	4.32
J. K.	Methionine	low protein	13.8	53.4	3.37	19.1	103.0	7.11
	None	low protein	14.6	54.7	3.73	20.3	123.9	7.25
	Methionine	adequate	10.0	63.3	4.11	10.4	96.4	5.33
L. L.	Methionine	low protein	12.1	50.6	3.25	18.2	92.0	5.75
	None	low protein	11.0	49.3	3.32	17.2	99.5	6.06
	Methionine	adequate	7.1	41.8	2.69	11.7	95.0	4.46
R. H.	Methionine	low protein	13.2	45.7	3.29	17.9	86.5	6.92
	None	low protein	10.6	36.4	3.06	19.2	102.1	6.98
	Methionine	adequate	9.9	34.6	2.65	13.3	86.8	6.44
D. D.	Methionine	low protein	7.7	38.1	3.32	15.2	87.8	5.50
	None	low protein	7.9	33.7	2.13	14.8	88.1	5.13
	Methionine	adequate	9.3	50.5	2.56	11.3	78.5	3.66
L. C. G.	Choline	low protein	13.5	64.5	3.63	18.7	86.0	5.85
	None	low protein	10.6	31.6	2.49	20.4	82.3	3.70
	Choline	adequate	9.0	47.6	1.98	12.4	85.5	4.68
K. W.	Choline	low protein	16.3	40.7	2.80	25.3	87.6	7.16
	None	low protein	14.4	30.9	2.50	22.5	83.0	6.56
	Choline	adequate	13.8	32.9	2.42	20.0	81.3	6.74
J. H. G.	Choline	low protein	9.4	28.5	1.73	23.7	74.0	5.06
	None	low protein	12.4	35.7	2.83	19.1	73.8	5.12
	Choline	adequate	7.1	29.7	1.67	10.9	70.3	4.19
G. B.	Choline	low protein	12.4	41.2	2.35	19.4	82.3	5.02
	None	low protein	10.3	39.4	2.17	14.4	78.7	4.20
	Choline	adequate	12.5	44.0	2.59	17.0	93.4	5.78
T. H.	Choline	low protein	12.3	33.6	2.67	17.9	84.0	6.62
	None	low protein	9.3	34.7	1.62	14.2	69.5	4.08
	Choline	adequate	11.0	39.1	2.88	17.0	127.9	8.43
J. H.	Choline	low protein	10.1	34.5	1.85	17.6	79.8	4.73
	Choline	adequate	9.0	44.8	2.99	11.0	69.7	3.04

$$\text{Specific Activity} = \frac{\text{Per cent of } P^{32} \text{ injected in Phospholipid} \times 100}{\text{mg. of phospholipid P}} \times 100$$

$$\text{Relative Specific Activity} = \frac{\text{Specific Activity of Phospholipid} \times 100}{\text{Specific Activity of Inorganic Phosphate}} \times 100$$

$$\text{Newly Formed Phospholipid} = \frac{\text{Per cent of } P^{32} \text{ injected in Phospholipids} \times 100}{\text{Specific Activity of Inorganic Phosphate}} \times 100$$

Neutral fats and fatty acids accumulate in the liver when there is a deficiency of the nitrogen base, choline, and of chemical groups which act as precursors—for instance, the methyl groups derived from methionine. The introduction into the diet of competitors for methyl groups—methyl acceptors—may also produce a decrease in phospholipid synthesis.[4] Both sequences of events have been produced in experimental animals maintained on diets low in proteins.

The Relation of Diet to Fatty Infiltration of the Liver in Rats and Human Subjects.—The time required to produce a fatty liver in rats by a diet containing only 5 per cent protein is seven days or less.[5] That the diet used in the human subjects (containing 7.6 per cent protein) would induce fatty infiltration of the liver in experimental animals is shown by the increased phosphorylation in the rats given methionine and choline after seven days on this diet. The present experiments indicate that, in the absence of other stress, a greater interval of time is necessary to produce a similar change in the normal human being maintained on a diet containing 32 Gm. of protein (7.6 per cent). Only one of twelve students who were given a single dose of choline or methionine after seven days on a low protein diet showed an increased rate of phospholipid turnover, as measured by the differences in specific and relative specific activities of the newly formed phospholipids. In the light of previous studies it would appear probable that very little fatty infiltration of the liver was present and that the administration of a low protein diet for seven days was not sufficient to produce detectable hepatic changes in otherwise normal human subjects.

SUMMARY

A study of phospholipid turnover employing radioactive phosphorus has shown that, in most normal adults, the phospholipid turnover shows no response to lipotropic agents following the administration of a low protein diet for seven days. Evidently the time interval required to produce hepatic changes in normal subjects is greater than this.

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RUTIN—ITS EFFECT ON THE EOSINOPHIL COUNT IN THE PERIPHERAL BLOOD OF NORMAL SUBJECTS

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THE mechanism of action of Rutin* (vitamin P) has never been clearly demonstrated. Indeed, doubt has recently been expressed that it has any vitamin-like properties at all.[1, 2]

This paper does not propose to discuss the vitaminic status of Rutin. Rather, its purpose is to offer evidence in regard to one of the theories proposed for its pharmacologic action.

The proposition that vitamin P might act through an "epinephrine-sparing mechanism"[3] to stimulate the pituitary and, through that gland's elaboration of ACTH, the adrenal cortex has been noted. In support of this theory are the findings of Hyman and Chambers[4] who noted that the adrenocortical hormones increase the strength of capillaries, and of Weinstein[5] who noted a parathyroid effect, increasing the resistance of connective tissue. The possible significance of such a finding was immediately apparent.

METHODS AND RESULTS

In an effort to demonstrate an adrenocorticotropic effect we utilized a modification of the Thorn (eosinophil) test,[6] substituting various dosages of Rutin for epinephrine. Both orally and intravenously administered Rutin were used for the determinations.

Subsequent to the initation of our studies, Clark and MacKay[1] reported that parenterally administered vitamin P "causes just as much decrease in adrenal ascorbic acid as epinephrine or pituitary adrenocorticotropic hormone." They feel that this may elicit a nonspecific stress or "alarm reaction" which might explain the reported physiologic and pharmacologic effects of vitamin P-like compounds.

In the light of the work of Gero,[7] who found a maximum urinary excretion of orally administered epicatechen, a flavone related to Rutin in one hour, it was felt that determinations should be done using graduated dosages of Rutin at intervals of one hour and four hours. Likewise two subjects were carried on 600 mg. of Rutin per day for two weeks, daily eosinophil counts being taken for a week prior to starting the drug and throughout the second week of treatment.

As evidenced by Table I, four one-hour tests done with dosages of 60 mg., two with 120 mg., two with 300 mg., and four with 600 mg. showed nothing approaching a significant 50 per cent reduction in the eosinophil count. Like-

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Received for publication, May 22, 1951.
*Kindly supplied to us by the Abbott Laboratories, North Chicago, Ill.

TABLE I, A. EOSINOPHIL COUNTS PRIOR TO AND ONE HOUR AFTER ORAL ADMINISTRATION OF RUTIN

DOSE	CONTROL	1 HR.
60 mg.	179	134
60 mg.	331	275
120 mg.	318	384
120 mg.	378	445
300 mg.	162	147
300 mg.	187	178
600 mg.	81	81
600 mg.	156	124
600 mg.	203	218
600 mg.	228	167
600 mg.	53	66
600 mg.	72	68

TABLE I, B. EOSINOPHIL COUNT PRIOR TO AND FOUR HOURS AFTER ORAL ADMINISTRATION OF RUTIN

DOSE	CONTROL	4 HR.
600 mg.	81	81
600 mg.	156	124
600 mg.	203	218
600 mg.	228	167
600 mg.	282	200
600 mg.	62	73
600 mg.	296	271

wise seven four-hour tests with dosages of 600 mg. produced no significant results. The two subjects given 600 mg. per day for two weeks (Table II) demonstrated statistically insignificant changes from the control series.

As shown in Table III, two subjects were given 50 mg. (2 ml.) of Rutin (Abbott) intravenously. Similarly, five persons were given 75 mg. (3 ml.) and another five 100 mg. (4 ml.). None of the above demonstrated a significant eosinophil response. Several control counts were done at fifteen minutes, thirty minutes, sixty minutes, and two hours to insure against an early and transient drop.

DISCUSSION

In view of the lack of a significant eosinophil response following the oral administration of Rutin, we might have concluded either that (1) there is no effect on the adrenal cortex, or (2) the compound is not absorbed in sufficient immediate quantity from the gastrointestinal tract to exercise an effect. Clark's findings with adrenal ascorbic acid levels would certainly appear to demonstrate adrenal cortical stimulation in the subjects used. In the light of these findings,

TABLE II. EOSINOPHIL COUNTS FIVE DAYS PRIOR TO AND FIVE DAYS FOLLOWING DAILY ORAL ADMINISTRATION OF 600 MG. OF RUTIN FOR TWO WEEKS

	CONTROL					2 WEEKS				
	1	2	3	4	5	1	2	3	4	5
Subject (1)	89	105	86	94	93	97	42	70	73	80
Subject (2)	73	139	144	87	100	144	119	125	117	124

TABLE III. EOSINOPHIL COUNTS PRIOR TO AND FOUR HOURS AFTER INTRAVENOUS ADMINISTRATION OF RUTIN

DOSE	CONTROL	4 HR.
50 mg.	134	133
	95	95
75 mg.	173	144
	151	165
	360	310
	109	87
	111	106
100 mg.	50	42
	100	91
	197	125
	89	58
	396	390

plus the remarkable correlation between the established capillary strengthening properties of the adrenocortical hormones[4] and the reported clinical effects of Rutin, it is difficult to understand our failure to elicit an eosinophil response to the intravenous administration of the compound. The point to be determined was not that Rutin, in exceedingly large doses, would produce a nonspecific stress—undoubtedly many compounds would do that—but whether or not therapeutic dosages of the drug would stimulate the adrenal cortex. We are forced to conclude that this paper presents evidence against a Pituitary-Target Gland Mechanism of action for Rutin, itself. We are not prepared to contravert the results of similar tests applied to others of the vitamin P-like group.

SUMMARY

A brief discussion of the "Pituitary-Target Gland Theory" for the pharmacologic action of Rutin is presented.

A modification of the (Thorn) eosinophil test is offered as a means of determining the adrenal-stimulating effect of Rutin.

Results of oral and parenteral administration of Rutin (Abbott) in both acute and chronic studies on the eosinophil count in normal subjects are presented.

It is concluded that Rutin, in therapeutic doses, does not exercise an adrenal cortical stimulating effect.

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STUDIES ON THE RELATIONSHIP OF TISSUE FLUID AND BLOOD LEVELS OF PENICILLIN

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THE partition of penicillin between the blood and tissue fluids is a problem which has attracted the attention of investigators from the time that this antibiotic agent first became available. While information concerning the concentration of drug in the blood is of great importance in studying its absorption and excretion, it may be of little or no value in ascertaining the actual quantity of antibacterial substance at a site of infection. There is little doubt that the "tissue" level of penicillin is related to the blood concentration, but the degree of relationship, particularly as it involves the duration of antibacterial effet, is not settled.

Investigations of the distribution of penicillin in various organs and body fluids of man and animals have shown that the drug can be found in the erythrocytes,[9] saliva and bile,[1] aqueous humor,[12] but does not appear at all or is present only in negligible quantities in tears,[9] pancreatic fluid,[1] and brain.[3] Cutting and his colleagues[3] and Richardson and associates[10] have shown that the largest quantity of penicillin is present in the kidneys after intravenous administration. Antibiotic applied topically in localized infections is removed so rapidly that the concentration after one to two hours is too low to prevent bacterial invasion.[5]

All studies in which comparisons between blood levels and organ content of penicillin have been made are open to the criticism that some of the drug in the tissues may have come from the blood. Such determinations, therefore, do not yield accurate information concerning the concentration of the antibiotic in the tissue fluid or cells. This objection has been met partially by investigations in which various body fluids have been examined. Thus, the penicillin content of the subcutaneous edema fluid and pleural and peritoneal effusions in nephrotics has been shown to equal that in the serum approximately two and one-half hours after administration.[2] Penicillin is present for a longer time in lymph than in blood, after intramuscular injection.[11] Study of infected wound exudates by Florey, Turton, and Duthie[4] after parenteral administration of the antibiotic, although complicated by the presence of penicillin-insensitive organisms, has revealed that the drug persists locally for a longer time than in the blood. Ungar[14] produced localized aseptic inflammatory reactions with turpentine, and demonstrated penicillin in the edema fluid of the lesions as long as five hours after it was given parenterally.

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The investigations described in this paper were supported by a grant from the Schenley Laboratories, New York, N. Y.

Received for publication, May 31, 1951.

The purpose of the present paper is to describe a new method for studying the distribution of penicillin in tissue fluids and to present evidence indicating that the drug persists for a longer time in tissue fluids than in blood.

METHODS

Preparation of Sterile Fibrin Clots.—A 10 per cent sterile solution of Fraction 1 of human plasma supplied by the Department of Physical Chemistry of the Harvard Medical School was made in 0.85 per cent saline by warming at 37° C. for two hours. One milliliter quantities of this were pipetted into sterile test tubes, 100 by 13 mm., and 30 to 50 units (in 0.05 ml. of saline) of sterile bovine thrombin (Parke-Davis) added to each tube. After gentle agitation, the mixtures were incubated at 37° C. (water bath) for thirty to forty-five minutes. The clots were removed aseptically by rimming with a sterile wire shaped to fit the test tube and placed in a sterile petri dish until used. By this method it was possible to make clots of uniform size and fibrin content and of a sufficient degree of firmness so that they could be handled with ease.

Implantation of Fibrin Clots Subcutaneously.—The rabbits used in this investigation were weighed prior to the beginning of each study and kept in the laboratory under constant supervision. After induction of light ether anesthesia and preparation of the shaved skin on the back, incisions about 2 cm. in length were made in several areas, the number varying with the experiment, and two to four sterile fibrin clots placed subcutaneously at each site, after which the wounds were closed with Michel clips.

Study of Clots After Removal.—At periodic intervals after the administration of penicillin intramuscularly, the wounds were opened, the fibrin clots removal under the strictest aseptic precautions, and ground in a sterile porcelain mortar. To the ground fibrin were added 1 ml. of a 2.5 per cent solution of trypsin (pH 7.5) previously sterilized by passage through a Seitz filter and 1 ml. of sterile broth. The mortar was then covered with sterile paper and placed in the incubator at 37° C. for forty-five minutes in order to allow complete digestion of the fibrin to take place. At the end of this time the penicillin content of the mixture was determined by the method of Rammelkamp.[8] Very little decrease in the volume of the fibrin mass occurred, even after it had been present in the subcutaneous tissues for appreciable periods of time. The total volume of fibrin, trypsin solution, and broth was 3 ml.; allowance for this dilution was made in every instance in calculating the quantity of penicillin in the original 1 ml. of clotted fibrin.

Penicillin Administration.—Crystalline penicillin G containing 1,594 units per milligram was used throughout this study, and solutions were freshly prepared before each experiment. All of the injections were made intramuscularly immediately after implantation of the fibrin clots and closure of the skin wounds. The quantity of antibiotic injected was varied with the weight and age of the animals because the rate of excretion was found to be higher in young animals. At periodic intervals after the injection of penicillin, blood was obtained aseptically from the ear vein at the same time as one or more fibrin clots were removed from under the skin, and both were assayed for drug content.

RESULTS

Penetration of Penicillin Into Fibrin Clots.—Sterile fibrin clots were prepared in the fashion described under Methods and placed in physiologic saline containing different quantities of penicillin. They were removed at thirty minutes, one hour, and two hours, rinsed in sterile saline, digested with trypsin, and assayed for penicillin. As can be seen from Table I, penetration of the fibrin clot was not rapid but, with all quantities of antibiotic ranging from 0.024 to 0.78 microgram per milliliter, about half the concentration in the surrounding medium was present in the fibrin clot after two hours of contact. Since these determinations were made by the serial dilution method of Rammel-

kamp,[8] these values represent only a one tube difference; this is within the range of experimental error. These data suggest, therefore, that even with fairly thick fibrin clots penicillin is almost completely equilibrated between the fibrin mass and the fluid menstruum with which it is in contact in about two hours. These results suggest that fibrin clots implanted under the skin take up penicillin present in the free tissue fluids and that, within certain limits, the drug content of these fibrin masses may be a valid indicator of the concentration of the antibiotic in the fluids surrounding them.

TABLE I. PENETRATION OF FIBRIN CLOTS BY PENICILLIN IN VITRO

AMOUNT OF PENICILLIN IN FLUID MEDIUM μG/ML.	AMOUNT OF PENICILLIN IN FIBRIN CLOT μG/ML. TIME OF CONTACT WITH PENICILLIN		
	30 MIN.	60 MIN.	120 MIN.
0.78	0.0185	0.075	0.39
0.195	0.009	0.0375	0.098
0.049	0.009	0.0185	0.024
0.024	0.0045	0.009	0.0185

The Effect of Trypsin on Penicillin.—In order to rule out the possibility that the tryptic digestion of the fibrin clot had a destructive effect on its penicillin content, an experiment was carried out to show that this enzyme had no effect on the drug. Mixtures of trypsin, in the same concentration as was used in the animal studies, were made with penicillin in saline solution and in fibrinogen; drug dissolved in saline without added trypsin was employed as a control. All of the solutions were assayed for penicillin after incubation at 37° C. for sixty minutes; a control was studied after one hour at 4° C. Contact with trypsin for sixty minutes at pH 7.5 and 37° C. did not decrease the content of antibiotic when compared to the untrypsinized control. These results validated the trypsin method of bringing the fibrin clots into solution.

Penicillin Content of Fibrin Clots and Blood After Single Intramuscular Injection of 10,000 Units.—Six rabbits were given a single intramuscular injection of 10,000 units of penicillin after the subcutaneous implantation of sterile fibrin clots. Blood levels were determined one, two, four, eight, and twelve hours after the administration of antibiotic, and the clots were removed and assayed for penicillin at the same time. The data presented in Table II show that none of the animals had detectable quantities of penicillin in the blood after two hours. The fibrin clots, on the other hand, contained assayable amounts of drug for four hours in four instances and for twelve hours in one animal. It is interesting to note that two hours after the administration of penicillin, the blood and clot levels were approximately the same in most cases, although in two the concentration in the fibrin was higher than that in the blood.

Penicillin Content of Fibrin Clots and Blood After Single Intramuscular Injections of 15,000 and 25,000 Units.—Six rabbits were injected intramuscularly with 15,000 units of penicillin, and the blood and fibrin clot penicillin content determined after one, two, four, eight, and twelve hours. Peni-

cillin was found to persist for from two to six hours longer in the fibrin clots than in the blood. Drug levels were higher in the fibrin than in the circulating blood, in some instances, two hours after the administration of penicillin. The data are presented in Table II.

Four animals were given a single dose of 25,000 units of penicillin. In them, persistence of antibiotic agent in the fibrin was from four to six hours longer than in the blood, and the clot levels after two hours exceeded those in the circulation. The data for this group are presented in Table II.

TABLE II. CONCENTRATION OF PENICILLIN IN FIBRIN CLOTS AND BLOOD FOLLOWING INJECTION OF 10,000 (6.25 MG.), 15,000 (9.375 MG.) AND 25,000 (15.625 MG.) UNITS

RABBIT NO.	WEIGHT (KG.)	PENICILLIN MG./KG. BODY WT.	PENICILLIN LEVELS—μG/ML.—TIME AFTER INOCULATION							
			BLOOD			FIBRIN CLOT				
			1 HR.	2 HR.	4 HR.	1 HR.	2 HR.	4 HR.	8 HR.	12 HR.
10,000 (6.25 mg.) Units										
1	2.75	2.27	0.049	0.024	0	--	0.0185	0.009	0	--
2	3.65	1.71	0.049	0.003	0	--	0.0045	0.0021	0.0021	0.0021
3	3.6	1.73	0.024	0.006	0	0.009	0.0185	0.0045	0	0
4	4.1	1.52	0.024	0.003	0	0.009	0.0045	0.0045	0	0
5	4.76	1.31	0.024	0	0	--	0.012	0.0045	0	0
15,000 (9.375 mg.) Units										
6	4.1	2.29	--	0.049	0	--	0.009	0.0045	0	--
7	3	3.13	--	0.006	0	--	0.0185	0.0185	0	--
8	3	3.13	0.098	0	0	--	0.0185	0.009	0	--
9	4.1	2.29	0.098	0	0	--	0.0185	0.0185	0	0
10	3	3.13	0.024	0.003	0	--	0.0045	0.0021	0.0021	--
11	3.4	2.76	0.098	0.024	0	0.0185	0.0185	0.009	0.0045	0
25,000 (15.625 mg.) Units										
12	3.2	4.9	0.024	0.003	0	--	0.009	0.0045	0.0045	0
13	3.2	4.9	--	0.003	0.0007	--	0.0045	0.0021	0.0012	0
14	3.4	4.6	0.049	0	0	0.012	0.012	0.0045	0	0
15	3.4	4.6	0.098	0.006	0	0.0375	0.0185	0.0375	0.0185	0

Penicillin Content of Fibrin Clots and Blood After Single Intramuscular Injections of 50,000 and 100,000 Units.—A single intramuscular injection of 50,000 units of penicillin was made in twenty-three rabbits after the subcutaneous implantation of sterile fibrin clots. Blood and clot levels of drug were determined simultaneously after one, two, four, eight, twelve, and twenty-four hours. As can be seen from Table III, penicillin was present in the circulation of twenty-two of the animals for only two hours after its administration. On the other hand, it was detectable for at least twelve hours in the fibrin clots in seventeen of the rabbits and for eight hours in all of those examined.

In fourteen rabbits studied after the injection of 100,000 units of penicillin intramuscularly (Table IV) the results were the same as those observed after the exhibition of 50,000 units, except that the antibiotic substance was still present in detectable quantities in the blood in eight animals after four hours.

TABLE III. CONCENTRATION OF PENICILLIN IN FIBRIN CLOTS AND BLOOD FOLLOWING INJECTION OF 50,000 UNITS (31.25 MG.)

RABBIT NO.	WEIGHT (KG.)	PENICILLIN MG./KG. BODY WT.	PENICILLIN LEVELS—μG/ML.—TIME AFTER INOCULATION								
			BLOOD			FIBRIN CLOT					
			1 HR.	2 HR.	4 HR.	1 HR.	2 HR.	4 HR.	8 HR.	12 HR.	24 HR.
1	3.8	8.23	0.098	0.003	0	0.009	0.009	0.0045	0.0022	0.0012	0
2	4	7.82	0.098	0.012	0	0.0045	0.0045	0.0045	0.0012	0	0
3	4.1	7.63	--	0.024	0	--	0.075	0.0375	0.009	0.0045	0
4	3	10.4	0.78	0.098	0	0.15	0.075	0.0375	0.0185	0.0045	0
5	3.2	9.78	0.39	0.049	0	0.075	0.075	0.0375	0.009	0	0
6	3	10.4	0.39	0.049	0	0.075	0.075	0.0375	0.009	0.0045	0
7	2.75	11.35	0.78	0.098	0	0.075	0.075	0.0375	0.0185	0.0045	0
8	2.75	11.35	0.78	0.049	0	0.075	0.075	0.0375	0.0185	0.009	0
9	3	10.4	0.195	0.024	0	0.0375	0.0375	0.0185	--	0.0022	0
10	2.75	11.35	0.195	0.024	0	0.0375	0.0185	0.0185	--	0.0022	0
11	2.75	11.35	0.195	0.024	0	0.0185	0.0185	0.0185	0.0045	0.0045	0
12	3	10.4	0.195	0.012	0	0.075	0.0375	0.0375	0.0185	0.009	0
13	3.2	9.78	0.195	0.012	0	0.075	0.075	0.0375	0.0185	0.009	0
14	2.75	11.35	0.098	0.024	0	0.185	0.0185	0.009	0.0045	0.0022	0
15	3.4	0.2	0.195	0.012	0	0.0185	0.0375	0.0185	0.009	0.0045	0
16	3.2	9.78	0.195	0.006	0	--	0.075	0.0375	0.0185	0.009	0
17	3	10.4	0.39	0.049	0.003	--	0.075	0.0375	0.0185	0.009	0
18	3.4	9.2	--	0.024	0	--	0.075	0.0375	0.009	0.009	0
19	3.2	9.78	--	0.098	0	--	0.15	0.0375	0.009	0.0045	0
20	3.86	8.1	0.195	0.049	0	0.0185	0.0185	0.0185	0.0045	0	0
21	3.4	3.2	0.39	0.049	0	0.0185	0.0375	0.0185	0.0045	0	0
22	2.6	12.05	0.098	0.006	0	0.075	0.0375	0.0185	0.0045	0	0
23	3.4	9.2	0.049	0.003	0	--	0.0185	0.009	0.0045	0	0

TABLE IV. CONCENTRATION OF PENICILLIN IN FIBRIN CLOTS AND BLOOD FOLLOWING INJECTION OF 100,000 UNITS (62.5 MG.)

RABBIT NO.	WEIGHT (KG.)	PENICILLIN MG./KG. BODY WT.	PENICILLIN LEVELS—μG/ML.—TIME AFTER INOCULATION							
			BLOOD			FIBRIN CLOT				
			2 HR.	4 HR.	8 HR.	2 HR.	4 HR.	8 HR.	12 HR.	24 HR.
1	3.4	18.4	0.049	0	--	0.15	0.075	0.0185	0.0012	0
2	3.4	18.4	0.024	0	--	0.075	0.0185	0.009	0	0
3	3.4	18.4	0.098	0.003	0	0.037	0.37	0.0045	0.0022	0
4	3	20.4	0.098	0.006	0	0.075	0.0375	0.009	0	--
5	3	20.4	0.049	0.006	0	0.075	0.0375	0.0185	0.009	0
6	3.4	18.4	0.012	0	--	0.15	0.075	0.0185	0.009	0
7	4.1	15.95	0.098	0.003	0	0.075	0.0375	--	0.0045	0
8	3	20.4	0.006	0.0015	0	0.0375	0.0185	0.0185	0.0045	0
9	2.75	22.35	0.049	0	--	0.0375	0.0185	0.0045	0	0
10	4.1	15.25	0.195	0.024	--	0.3	0.15	0.375	0.0185	0
11	4.1	15.25	0.024	0.003	0	0.075	0.0375	0.0375	0.009	0
12	2.75	22.35	0.024	0.0015	0	0.075	0.0375	0.0185	0.0045	0
13	3.3	18.95	0.024	0	--	0.075	0.0375	0.009	0.0045	0
14	4.1	15.25	0.049	0	--	0.075	0.0375	--	0.009	0

DISCUSSION

A new method for estimating the level of penicillin in tissue fluids has been devised. This consists of the subcutaneous implantation, in rabbits, of sterile fibrin clots of uniform composition and the determination of drug in these clots, after trypsin digestion, at specific intervals following intramuscular injection. This technique appears to offer a more accurate quantitative method of estimating the tissue fluid level of penicillin than study of infected exudates, serous collections resulting from irritants, or pieces of various organs and tissues, since it eliminates contamination with blood, bacteria, excessive amounts of lymph, and the products of inflammation.

This procedure allows an approximation of the quantity of penicillin in subcutaneous tissue fluids. This is borne out by the observation that immersion of fibrin clots of the same composition as those implanted under the skin in solutions of penicillin leads to equilibration of the drug between the fibrin mass and the surrounding menstruum in about two hours, to a degree within the limits of experimental error inherent in the method of assay. These results confirm those of Nathanson and Liebhold,[7] who showed that penicillin diffuses into fibrin as well as in agar. Masonyi and associates[6] found that this antibiotic agent penetrated fibrin only to a limited extent, thick layers being impermeable. It is possible that maximal diffusion of penicillin was not obtained in the experiments described in this paper. If this is so, it may have been due to the use of a high concentration of fibrinogen. Clots containing smaller quantities of fibrin were studied but were discarded because they contracted so much after implantation in the subcutaneous tissues for twelve hours or longer that quantitative determinations of drug were impossible.

The procedures necessary to bring the fibrin clots into complete solution have been shown to be without harm to penicillin. Contact of saline solutions of known quantities of penicillin with trypsin, under the same conditions as those present in the experiments, produced no decrease in antibacterial activity.

These studies indicate that penicillin is present in tissue fluids for a longer time than in the blood after intramuscular injection. Peak levels of drug usually appeared in the fibrin clots in about two hours after parenteral administration and were, in many instances, greater than the highest concentrations demonstrable in the circulation. A direct relationship exists between the amount of penicillin given intramuscularly and both the intensity and duration of antibacterial effect in the fibrin clots or, by inference, in the subcutaneous tissue fluids. Thus, when 15,000 units of penicillin were injected, the drug was detectable for from two to six hours longer in the fibrin clots than in the blood, while with 50,000 to 100,000 units, tissue fluid concentrations were present for from six to ten hours longer than in the peripheral circulation. These observations confirm those of Florey and co-workers,[4] Ungar,[14] and Schachter.[11]

The clinical implications of the data presented in this paper are readily apparent. The fact that penicillin is capable of penetrating fibrin has been proved in human disease by the successful control of infections in which

fibrinous exudates constitute a major feature, e.g., empyema, bacterial meningitis, and subacute and acute bacterial endocarditis. The persistence of penicillin activity in fibrin masses for longer periods of time than in the blood emphasizes the fact that the quantity of drug in the circulation has significance only because it is related to the intensity and duration of the antibacterial activity in the tissues. Since penicillin can be found for considerably longer periods of time in tissue fluids and fibrin masses than in the blood, the clinical use of so-called "discontinuous" therapy appears to be entirely reasonable. The data obtained in these studies validate, on an experimental basis, the clinical observations that the administration of relatively large quantities of penicillin at infrequent intervals (e.g., every eight to twelve hours) is very effective in eliminating infections due to penicillin-sensitive organisms, in spite of the fact that blood levels are present for only short periods of time.

CONCLUSIONS

1. A new technique for the study of the penicillin content of tissue fluids in the absence of blood, excessive quantities of lymph, and the products of inflammation has been devised.

2. Penicillin persists for a longer time in fibrin and probably in tissue fluids than in the circulating blood.

3. There is a direct relationship between the quantity of penicillin administered intramuscularly and the intensity and duration of antibacterial activity in tissue fluids.

4. The data presented offer experimental support for the "discontinuous" type of therapy in infections due to penicillin-sensitive organisms.

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CROSS-RESISTANCE TO ANTIBIOTICS

Effect of Repeated Exposure of Bacteria to Aureomycin, Terramycin, Chloramphenicol or Neomycin on the Resistance to All of These Antibiotics and to Streptomycin and Penicillin

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THE development of increased resistance of bacteria in the course of exposure to antibiotics, both in vitro and during therapy, is well recognized. Although the exact mechanism whereby resistance comes about may still be a matter of controversy, the importance of this phenomenon in the treatment of infections is generally appreciated. The clinician faced with an infection by an organism which has become resistant in the course of antibiotic therapy and before that infection has been eradicated may have to resort to other agents. It is, therefore, important to know whether the resistance so acquired is limited to the agent to which the organism had been exposed or extends to other antibiotics which may then likewise prove ineffective for continuing the therapy.

The question of cross-resistance among antibiotics may have more fundamental implications. The mechanisms whereby effective antibiotics exert their antimicrobial action are not established. It seems likely, however, that the acquisition of resistance is intimately related to these modes of action. Furthermore, the introduction of antibiotics with different chemical and physical properties but with wide and very similar antimicrobial spectra suggests that such antibiotics may exert their action, at least in part, along some common metabolic pathways. It is, therefore, of additional interest to determine whether cross-resistance develops among these related antibiotics. Likewise, the development of strains dependent for growth on any given antibiotic may offer an opportunity to determine whether the growth promoting property of that antibiotic could be supplied by other agents.

In the present study an attempt was made to increase the resistance of a number of organisms to each of four antibiotics, namely, aureomycin, terramycin, chloramphenicol, and neomycin, by repeated subculture on increasing concentrations of the individual agents. These organisms and the same strains subcultured on the same media but in the absence of antibiotics were then tested for sensitivity to all four agents. Some of the organisms were also tested with penicillin and streptomycin. In the course of this study a chloramphenicol-dependent variant of one of the organisms was developed.[1] The effect of the other antibiotics on the growth of this strain was also studied.

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Aided by a grant from the United States Public Health Service.
Received for publication, July 5, 1951.

MATERIALS AND METHODS

Organisms Used.—The strains of organisms used in this study and their sources are listed in Table I. Except for the enteric pathogens, all of these strains had recently been isolated from patients on the wards of the Boston City Hospital. None of these patients had any known previous exposure to antibiotics. Cultures, originally picked from individual colonies, had been subcultured on antibiotic-free media only a few times before the study was begun.

Media.—Heart infusion agar (Difco), pH ± 7.4, was used throughout for both the antibiotic-free and antibiotic-containing media. In addition, 10 per cent defibrinated equine blood was added throughout this study to the agar used for the cultivation of each of the last seven organisms listed in Table I, but not for the first seven strains. Brain heart infusion broth (Difco), pH ± 7.4, was used for dilution of cultures and antibiotics.

Antibiotics.—*Crystalline aureomycin hydrochloride* in sterile vials containing 100 mg. each was supplied by Dr. Stanton M. Hardy of Lederle Laboratories. Chloramphenicol (Chloromycetin) was supplied by Dr. E. A. Sharp of Parke, Davis & Company in vials containing 250 mg. of the dried powder. Crystalline terramycin hydrochloride was supplied by Dr. Gladys L. Hobby of Charles Pfizer & Co., Inc. Neomycin sulfate was furnished by Dr. Lawrence W. Smith of Commercial Solvents Corporation in vials, each of which contained 115,000 units and had a labelled potency of 173 units per milligram. Stock solutions of each of these antibiotics, except aureomycin, were prepared and distributed in 1 to 2 ml. amounts into vials which were then sealed and stored in a dry ice box at −70° C. Individual vials were opened and used at the time of each transfer. Solutions of aureomycin, however, were freshly prepared each time immediately before they were used. Commercial preparations of crystalline potassium penicillin G (Commercial Solvents Corp., 1,595 units per milligram) and streptomycin (calcium chloride complex, Merck) were used for the tests with these antibiotics. The concentrations of all antibiotics are expressed in micrograms of the active substance per milliliter of medium.

TABLE I. ORGANISMS USED IN THIS STUDY

ORGANISM	SOURCE	DIAGNOSIS
Escherichia coli communis	Urine	Pyelonephritis
Aerobacter aerogenes	Urine	Pyelonephritis
Klebsiella pneumoniae, type A	Urine	Pyelonephritis and Meningitis*
Salmonella typhi	C. D. C.†	
Salmonella paratyphi, B	C. D. C.†	
Shigella sonnei, Duval	C. D. C.†	
Staphylococcus aureus, 1‡	Pus	Otitis media
Pneumococcus, type 1	Sputum	Pneumonia
Pneumococcus, type 3	Spinal Fluid	Meningitis
Streptococcus hemolyticus,§	Throat swab	Pharyngitis
Streptococcus viridans	Blood	Subacute bacterial endocarditis
Enterococcus (Group D)	Pus	Infected head wound
Hemophilus influenzae, type B	Spinal Fluid	Meningitis
Staphylococcus aureus, 2‡	Pus	Infected amputation stump

*The same organism was also obtained from blood and spinal fluid.

†Communicable Disease Center, Chamblee, Ga. Supplied by Drs. P. R. Edwards and W. H. Ewing.

‡Hemolytic, coagulase positive.

§Group A, type M30.

Tests for Sensitivity.—The serial plate-dilution test was used. Since the cultures to be tested were grown on solid media, the inoculum was prepared in each instance by scraping off some of the surface growth with a 3 mm. loop and emulsifying the harvest in 0.2 ml. of broth. Serial twofold dilutions of the four antibiotics in the agar (with or without blood, as already noted) were prepared in appropriate ranges to include both good growth and complete inhibition of all the strains. A 1 ml. loopful of the emulsion of the organisms was then inoculated on each of these series of plates and on a plate of

antibiotic-free medium, the latter serving as a control of growth. The plates were incubated at 37° C. for forty-eight hours, and the amount of growth was then noted with the aid of an engraver's lens (3×). The sensitivity was expressed as the minimum concentration of antibiotic on which complete inhibition was recorded, as determined by the failure to recognize any discernible growth. Viable organisms, however, were frequently demonstrable by subculture on antibiotic-free media from plates which contained concentrations of antibiotic from one to several dilutions beyond this end point.

Development of Resistant Strains.—Five series of subcultures of each strain were carried out in parallel, one series on antibiotic-free medium, and one each on media con-

TABLE II. CHANGES IN RESISTANCE OF BACTERIA TO AUREOMYCIN AFTER REPEATED SUBCULTURES ON ANTIBIOTIC-FREE AGAR AND ON AGAR CONTAINING PROGRESSIVELY INCREASING CONCENTRATIONS OF AUREOMYCIN, TERRAMYCIN, CHLORAMPHENICOL, AND NEOMYCIN

	MINIMUM COMPLETE INHIBITING CONCENTRATION OF AUREOMYCIN, μG. PER ML.					
	BEFORE SUB-CULTURE	AFTER 40 SUBCULTURES† ON AGAR CONTAINING:				
ORGANISM		NO ANTIBIOTIC	AUREOMYCIN	TERRAMYCIN	CHLORAMPHENICOL	NEOMYCIN
Escherichia coli	50	50	6400	400	200	50
Aerobacter aerogenes	100	100	200	1600	200	50^{13}
Klebsiella pneumoniae, A	25	50	800	200	200^{30}	25^{24}
Salmonella typhi	50	25	200	200	200	50
Salmonella paratyphi B	50	50	1600	1600	200	50^{22}
Shigella sonnei	50	25	50	100^{11}	100	50
Staphylococcus aureus, 1	12.5	25	50	200	50	12.5
Pneumococcus, type 1	1.6	1.6	3.1	6.3	0.8	1.6^{14}
Pneumococcus, type 3	1.6	1.6	3.1	12.5	1.6	6.3^{30}
Streptococcus hemolyticus	6.3	1.6	50*	6.3	3.1	3.1
Streptococcus viridans	25	12.5	200	50	6.3	3.1^{26}
Enterococcus	25	25	200	100	12.5	50^{12}
Hemophilus influenzae, B	12.5	12.5	12.5	12.5	12.5	12.5
Staphylococcus aureus, 2	12.5	12.5	25	100	6.3	12.5^{36}

*This strain was classified as a group B beta hemolytic streptococcus.
†Superscripts indicate the number of subcultures when they were less than forty.

TABLE III. CHANGES IN RESISTANCE OF BACTERIA TO TERRAMYCIN AFTER REPEATED SUBCULTURES ON ANTIBIOTIC-FREE AGAR AND ON AGAR CONTAINING PROGRESSIVELY INCREASING CONCENTRATIONS OF TERRAMYCIN, AUREOMYCIN, CHLORAMPHENICOL, AND NEOMYCIN

	MINIMUM COMPLETE INHIBITING CONCENTRATION OF TERRAMYCIN, μG. PER ML.					
	BEFORE SUB-CULTURE	AFTER 40 SUBCULTURES* ON AGAR CONTAINING:				
ORGANISM		NO ANTIBIOTIC	TERRAMYCIN	AUREOMYCIN	CHLORAMPHENICOL	NEOMYCIN
Escherichia coli	12.5	12.5	400	200	100	12.5
Aerobacter aerogenes	50	100	3200	200	100	6.3^{13}
Klebsiella pneumoniae, A	12.5	6.3	800	200	200^{30}	6.3^{24}
Salmonella typhi	12.5	12.5	400	200	100	6.3
Salmonella paratyphi B	25	12.5	800	400	100	6.3^{22}
Shigella sonnei	6.3	3.1	200^{11}	100	25	3.1
Staphylococcus aureus, 1	6.3	6.3	400	100	12.5	6.3
Pneumococcus, type 1	0.8	0.4	12.5	6.3	0.8	0.2^{14}
Pneumococcus, type 3	0.8	1.6	25	6.3	0.8	1.6^{30}
Streptococcus hemolyticus	1.6	0.8	12.5	100†	3.1	1.6
Streptococcus viridans	3.1	3.1	50	400	3.1	1.6^{26}
Enterococcus	25	50	400	400	12.5	25^{12}
Hemophilus influenzae, B	3.1	3.1	25	50	3.1	1.6
Staphylococcus aureus, 2	6.3	6.3	400	50	6.3	3.1^{36}

*Superscripts indicate the number of subcultures when they were less than forty.
†This strain classified serologically as group B.

TABLE IV. CHANGES IN RESISTANCE OF BACTERIA TO CHLORAMPHENICOL AFTER REPEATED SUBCULTURES ON ANTIBIOTIC-FREE AGAR AND ON AGAR CONTAINING PROGRESSIVELY INCREASING CONCENTRATIONS OF CHLORAMPHENICOL, AUREOMYCIN, TERRAMYCIN, AND NEOMYCIN

	MINIMUM COMPLETE INHIBITING CONCENTRATION OF CHLORAMPHENICOL, μG./ML.					
		AFTER 40 SUBCULTURES* ON AGAR CONTAINING:				
ORGANISM	BEFORE SUBCULTURE	NO ANTIBIOTIC†	CHLORAMPHENICOL	AUREOMYCIN	TERRAMYCIN	NEOMYCIN
Escherichia coli	12.5	6.3 (12.5^{14})	1600	200	25	3.1
Aerobacter aerogenes	12.5	50 (25^{14})	1600	50	50	3.1^{13}
Klebsiella pneumoniae, A	6.3	50 (6.3^{14})	1600^{30}	100	50	0.4^{24}
Salmonella typhi	6.3	12.5 (12.5^{14})	800	25	50	1.6
Salmonella paratyphi B	12.5	100 (12.5^{14})	6400	200	200	1.6^{22}
Shigella sonnei	3.1	50 (3.1^{14})	50	100	50^{11}	1.6
Staphylococcus aureus, 1	25	100 (25^{14})	200	50	100	25
Pneumococcus, type 1	1.6	0.8 (1.6^{14})	1.6	3.1	3.1	0.4^{14}
Pneumococcus, type 3	1.6	1.6 (1.6^{14})	3.1	3.1	3.1	3.1^{30}
Streptococcus hemolyticus	3.1	1.6 (3.1^{14})	6.3	6.3‡	6.3	3.1
Streptococcus viridans	6.3	3.1 (6.3^{14})	25	3.1	3.1	0.8^{26}
Enterococcus	25	25 (25^{14})	1600	25	25	6.3^{12}
Hemophilus influenzae, B	1.6	3.1 (1.6^{14})	1.6	3.1	0.8	0.4
Staphylococcus aureus, 2	12.5	25 (25^{14})	400	25	50	1.6^{36}

*Superscripts indicate the number of subcultures when they were less than forty.

†Because of the discrepancies in some of these values, the sensitivity of each strain after the 14th transfer is also shown.

‡This strain classified serologically as group B.

TABLE V. CHANGES IN RESISTANCE OF BACTERIA TO NEOMYCIN AFTER REPEATED SUBCULTURES ON ANTIBIOTIC-FREE AGAR AND ON AGAR CONTAINING PROGRESSIVELY INCREASING CONCENTRATIONS OF NEOMYCIN, AUREOMYCIN, TERRAMYCIN, AND CHLORAMPHENICOL

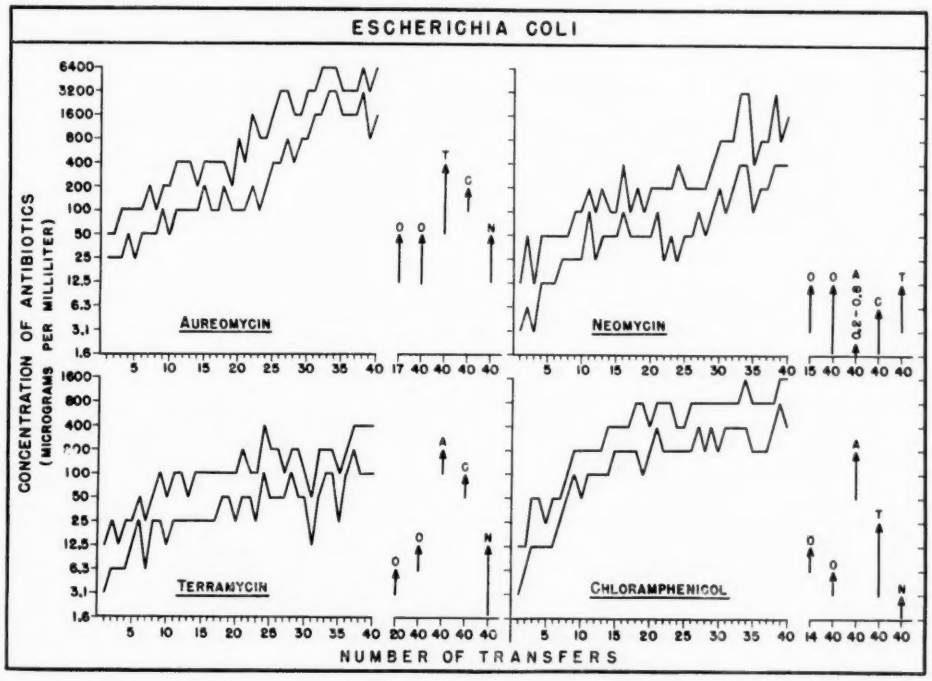
	MINIMUM COMPLETE INHIBITING CONCENTRATION OF NEOMYCIN, μG./ML.					
		AFTER 40 SUBCULTURES* ON AGAR CONTAINING:				
ORGANISM	BEFORE SUBCULTURE	NO ANTIBIOTIC	NEOMYCIN	AUREOMYCIN	TERRAMYCIN	CHLORAMPHENICOL
Escherichia coli	12.5	12.5	1600	0.8	12.5	6.3
Aerobacter aerogenes	12.5	3.1	400^{13}	3.1	3.1	1.6
Klebsiella pneumoniae, A	6.3	0.4^{15}	200^{24}	0.8	0.8	1.6^{30}
Salmonella typhi	25	3.1	400	0.8	1.6	1.6
Salmonella paratyphi, B	100	25	12,800^{22}	12.5	25	25
Shigella sonnei	25	50	400	200	200^{11}	6.3
Staphylococcus aureus, 1	25	25	800	50	50	100
Pneumococcus, type 1	200	100	12,800^{14}	100	800	100
Pneumococcus, type 3	200	100	3,200^{30}	25	200	200
Streptococcus hemolyticus	200	200	3,200	50†	100	50
Streptococcus viridans	400	400	25,600^{26}	200	400	400
Enterococcus	200	200	25,600^{12}	100	400	100
Hemophilus influenzae, B	6.3	6.3	100	--	3.1	3.1
Staphylococcus aureus, 2	6.3	6.3	6,400^{36}	12.5	6.3	6.3

*Superscripts indicate the number of transfers when they were less than for y.

†This strain was classified as group B.

taining aureomycin, terramycin, chloramphenicol, and neomycin. Each subculture of the four antibiotic series was carried out as in the sensitivity tests; the inoculum for subculture was obtained in each instance from the plate containing the maximum concentration of the same antibiotic which permitted good growth (either confluent colonies or

numerous well developed colonies).* Each subculture was grown for forty-eight hours at 37° C. A total of forty such subcultures were carried out in all but a few instances. The last subculture of each of these five series was then tested for sensitivity to each of the four antibiotics. In addition some of the gram-positive coccal strains were tested for sensitivity to penicillin, and most of the organisms which had been exposed to neomycin and their corresponding controls were tested with streptomycin.



Explanation of Figs. 1 to 4.—The upper lines connect the minimum complete inhibiting concentrations of the antibiotic indicated, and the lower lines connect the corresponding values for the maximum concentration of that antibiotic which supported good growth at each of the successive transfers. Each arrow represents the minimum complete inhibiting concentration (head) of the designated antibiotic and the corresponding maximum concentration supporting good growth (lowest point) of the organism after the indicated number of subcultures on antibiotic-free media (*O*) or on media containing aureomycin (*A*), terramycin (*T*), chloramphenicol (*C*), or neomycin (*N*).

Fig. 1.—Changes in resistance and cross-resistance induced in a strain of *Escherichia coli* as a result of repeated subcultures on agar containing increasing concentrations of each of four antibiotics.

RESULTS

In Table II are listed the minimum complete inhibiting concentrations of each of the fourteen strains at the start of the experiment, after the serial subcultures on antibiotic-free media and after their transfer serially on media containing increasing concentrations of the four antibiotics under study, namely, aureomycin, terramycin, chloramphenicol, and neomycin. The corresponding values for the sensitivity of each of the strains to terramycin, chloramphenicol,

*This offered a large number of organisms for the inoculum and was most likely to yield growth on subculture. In two experiments, one with the twentieth transfers on aureomycin and the other with the twenty-first transfers on terramycin, attempts were made to determine whether an inoculum from the plate showing the least discernible growth would yield more resistant organisms on subculture. Although the inocula of corresponding strains were obtained from plates containing two to thirty-two fold concentrations of antibiotic, the sensitivity of the subcultures was the same in each instance.

and neomycin are listed in Tables III, IV, and V, respectively. The progressive changes in the resistance of some of the individual strains that occurred in the course of the serial subcultures on single antibiotics are shown in Figs. 1 to 4. These figures show both the minimum complete inhibiting concentrations, as here defined, and the maximum concentrations which supported good growth of the organisms at each of the transfers in the homologous antibiotic. They also show, for comparison, the corresponding values for the same strain after it had been transferred in antibiotic-free media and in media containing progressively increasing concentrations of the other three antibiotics. For simplicity of presentation, the results will be summarized separately with respect to each of the antibiotics.

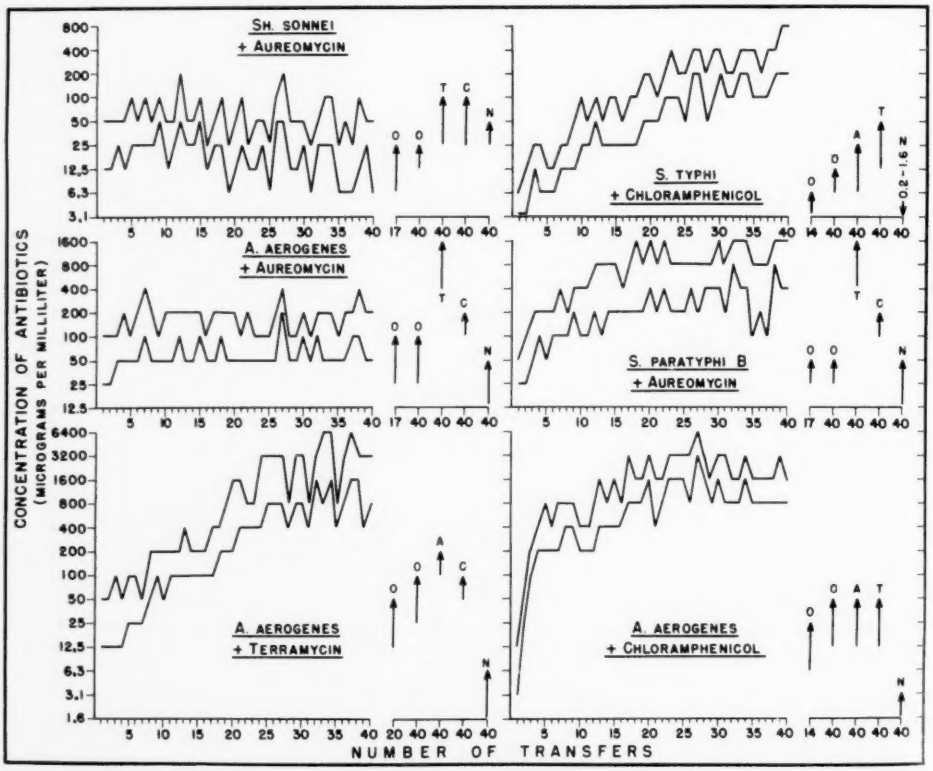


Fig. 2.—Changes in resistance of some gram-negative bacilli resulting from repeated subcultures on antibiotic-containing agar.

Changes in Resistance to Aureomycin.—From Table II it is seen that the fourteen strains had retained their original sensitivity to aureomycin, within the limits of the test, during forty subcultures on antibiotic-free media. There was considerable individual variation among these strains, however, with respect to their development of resistance during similar subcultures on aureomycin-containing agar. Of the seven gram-negative bacilli, only four showed signifi-

cant increases in resistance, ranging from eight to one hundred twenty-eight fold, while the sensitivity of *A. aerogenes*, *Sh. sonnei* and *H. influenzae* remained essentially unchanged. Of the seven gram-positive coccal strains only the streptococci increased in resistance,* while the two staphylococcal and the two pneumococcal strains retained their original sensitivity to aureomycin during the forty transfers in that antibiotic.

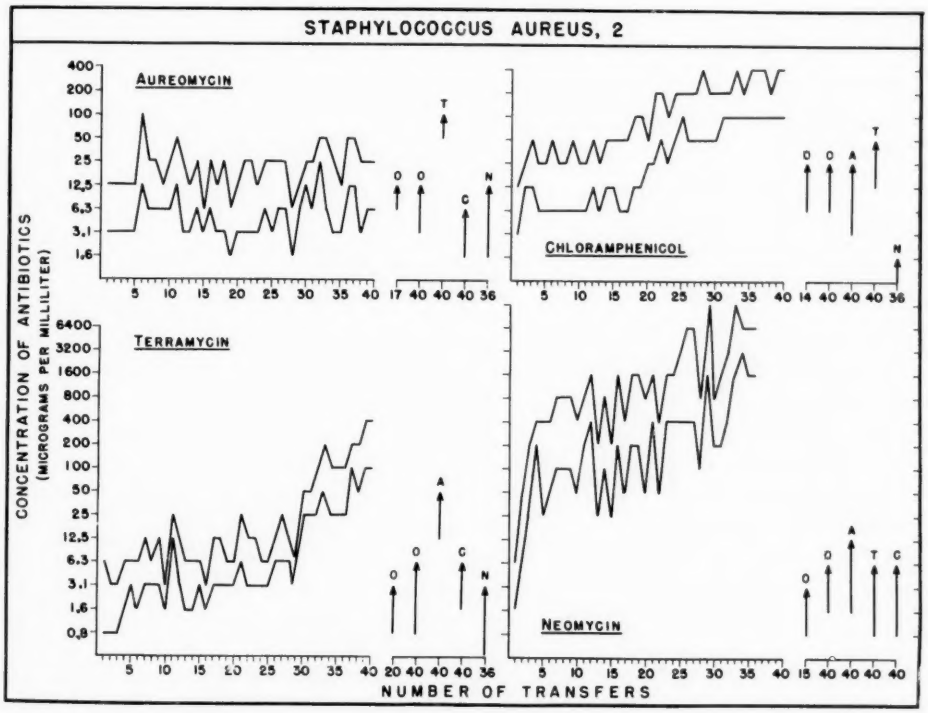


Fig. 3.—Changes in resistance and cross-resistance induced in a strain of *Staphylococcus aureus* as a result of repeated subcultures on blood agar containing increasing concentrations of each of four antibiotics.

All the strains which had been subcultured forty times on terramycin, with the exception of *H. influenzae* showed moderate to marked increases in resistance to aureomycin. In fact, only five of the fourteen strains showed increases in resistance to aureomycin following the repeated exposures to terramycin, which were of lesser magnitude than those which followed the repeated transfers on the homologous agent. All of these fourteen strains also showed moderate or marked increases in resistance to terramycin as shown in Table III.

The changes in aureomycin sensitivity following exposure to chloramphenicol varied with the strains. The gram-negative bacilli, other than *H. influenzae*, increased in resistance to aureomycin either to the same extent as they did after exposure to the homologous agent, or less so. On the other hand, *H.*

*The group A hemolytic streptococcus showed no change (or possibly a twofold increase) in resistance during the first thirty-two subcultures on aureomycin. The serologic reaction of the fortieth passage strain renders its identity with the original group A strain doubtful.

influenzae and all of the gram-positive cocci, with the possible exception of *S. aureus*,1, either remained unchanged or showed an apparent increase in sensitivity to aureomycin after the repeated exposures to chloramphenicol.

All of the strains which had been repeatedly transferred on neomycin and which increased markedly in resistance to that antibiotic (Table V) showed no change in susceptibility to aureomycin.

Further details concerning changes in aureomycin resistance in eight of the strains are shown in the appropriate portions of Figs. 1 to 4. These figures also show the values for the maximum concentrations of aureomycin which permitted good growth of the strains at the time of each subculture. The changes in these values corresponded very closely to those of the minimum complete inhibiting concentrations which are listed in the tables.

Changes in Terramycin Resistance.—(Table III.) The sensitivity of the organisms to terramycin did not change significantly during repeated subcultures on antibiotic-free media. During the transfers on terramycin, all fourteen strains increased in resistance eight to sixty-four fold, the increases being only slightly less with the gram-positive cocci and *H. influenzae* than in the coliform and enteric bacilli.

Similar subcultures on aureomycin-containing media also resulted in increased resistance to terramycin. In most instances this increase was only slightly less than that which resulted from exposure to the homologous agent, but the various streptococcal strains showed the same or greater increases.

Repeated exposures to chloramphenicol yielded variable results. In this instance the coliform and enteric bacilli increased in resistance to terramycin, but less so than did the organisms which had been transferred on the homologous agent or on aureomycin. The gram-positive cocci and *H. influenzae* showed no changes in their sensitivity to terramycin as a result of their repeated exposures to chloramphenicol.

Organisms made resistant to neomycin by repeated subculture on that antibiotic showed essentially no change in their sensitivity to terramycin.

The terramycin sensitivity of three of the strains at the time of each of the subcultures on that antibiotic and after the repeated transfers on antibiotic-free media or on media containing the three other antibiotics are shown in Figs. 1, 2, and 3. The maximum concentration of terramycin which supported growth is also shown in each instance.

Changes in Resistance to Chloramphenicol.—(Table IV and Figs. 1 to 4.) Repeated subcultures on chloramphenicol yielded variable results. The coliform organisms, two of the enteric bacilli, two of the streptococcal strains and *Staphylococcus 2* all showed considerable increases in resistance to the homologous agent. The other six strains showed only slight increases in resistance or changes which were difficult to evaluate because of apparent differences in the sensitivity of the corresponding control strains that had been transferred on antibiotic-free media and tested at about the same time.

Repeated exposures to aureomycin or terramycin resulted in only minor increases in resistance to chloramphenicol, except for the strain of *E. coli* which showed a considerably increased resistance to chloramphenicol after the expos-

ures to aureomycin. The development of increased resistance of all of the strains to neomycin which resulted from their repeated subcultures on that agent (Table V) failed to affect the sensitivity of most of them to chloramphenicol and even appeared to increase the sensitivity of some of the strains to the latter antibiotic.

Resistance to Neomycin.—(Table V and Figs. 1, 3, and 4.) The results obtained with neomycin were the most uniform and definitive. All of the strains increased moderately or markedly in resistance during the subcultures in this agent, and this increase usually occurred more rapidly than with the other three agents. In some instances the strains increased in resistance to neomycin so rapidly that they grew freely on the maximum convenient concentration

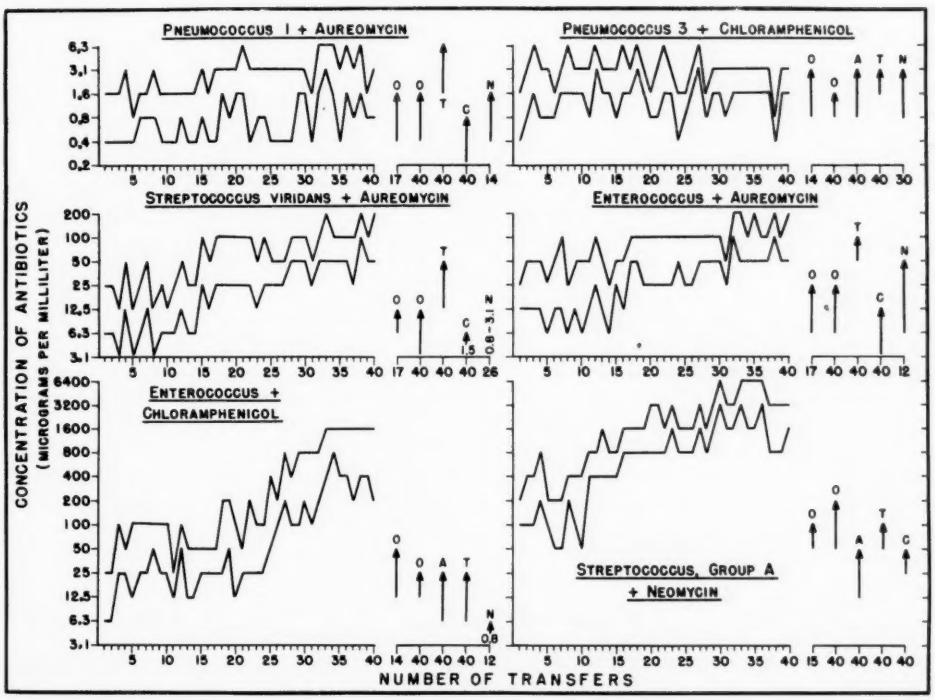

PNEUMOCOCCUS 1 + AUREOMYCIN
PNEUMOCOCCUS 3 + CHLORAMPHENICOL
STREPTOCOCCUS VIRIDANS + AUREOMYCIN
ENTEROCOCCUS + AUREOMYCIN
ENTEROCOCCUS + CHLORAMPHENICOL
STREPTOCOCCUS, GROUP A + NEOMYCIN
CONCENTRATION OF ANTIBIOTICS (MICROGRAMS PER MILLILITER)
NUMBER OF TRANSFERS

Fig. 4.—Changes in resistance of some gram-positive cocci resulting from repeated subcultures on blood agar containing various antibiotics.

(6.3 mg. per milliliter) after only a few transfers, and this was readily reproduced by starting again with the control strains. The corresponding strains which had been repeatedly transferred on antibiotic-free media showed no increase in resistance to neomycin. The same was true, in almost every instance, after the organisms had been repeatedly subcultured on either aureomycin, terramycin, or chloramphenicol.

Effect of Penicillin Sensitivity.—(Table VI.) Five of the gram-positive coccal strains were tested for sensitivity to penicillin after serial transfers on antibiotic-free blood agar and on similar media containing each of four anti-

TABLE VI. PENICILLIN SENSITIVITY OF FIVE STRAINS OF ORGANISMS SUBJECTED TO SERIAL SUBCULTURES ON AGAR WITH AND WITHOUT FOUR OTHER ANTIBIOTICS

ORGANISM	MINIMUM COMPLETE INHIBITING CONCENTRATION OF PENICILLIN, μG./ML. AFTER 40 SUBCULTURES* ON AGAR CONTAINING:				
	NO ANTIBIOTIC	AUREOMYCIN	TERRAMYCIN	CHLORAMPHENICOL	NEOMYCIN
Pneumococcus, type 1	0.02	0.01	0.01	0.01	0.02^{14}
Pneumococcus, type 3	0.04	0.02	0.01	0.02	--
Streptococcus hemolyticus	0.02	0.02	0.01	0.01	0.01
Streptococcus viridans	0.05	0.05	0.05	0.05	0.05^{26}
Enterococcus	6.3	6.3	6.3	6.3	6.3^{12}

*When less than 40 subcultures were made, the number of transfers is shown in the superscripts.

biotics—aureomycin, terramycin, chloramphenicol, and neomycin. The sensitivity of each of these strains to penicillin was the same after each of these procedures.

Effect of Development of Neomycin Resistance on the Sensitivity to Streptomycin.—The strains which had become resistant to neomycin after serial subcultures on that antibiotic and the corresponding control strains which had been transferred repeatedly on antibiotic-free media were tested simultaneously for their sensitivity to streptomycin. The results are shown in Table VII. Only the pair of strains of Shigella were inhibited by the same concentration of streptomycin. The other twelve strains that were tested, each of which had increased in resistance to neomycin (as shown in Table V), also showed greater resistance to streptomycin than did the corresponding control strains which had not been exposed to any antibiotics. In most of the strains this decrease in sensitivity to streptomycin was not so great as to neomycin, but with some of them it was of similar magnitude.

Development of a Chloramphenicol-Dependent Variant.—During the course of this study it was noted that the strain of *Klebsiella pneumoniae* after several serial transfers on chloramphenicol failed to grow when cultured on antibiotic-free agar. All of the other strains of this and the other series apparently grew

TABLE VII. COMPARISON OF SENSITIVITY TO STREPTOMYCIN OF STRAINS SUBJECTED TO SERIAL SUBCULTURES ON AGAR WITH AND WITHOUT NEOMYCIN

ORGANISM	MINIMUM COMPLETE INHIBITING CONCENTRATION OF STREPTOMYCIN IN MICROGRAMS PER MILLILITER AFTER 40 SUBCULTURES* IN AGAR CONTAINING:	
	NO ANTIBIOTIC	NEOMYCIN
Escherichia coli	25	400
Aerobacter aerogenes	6.3	200^{13}
Klebsiella pneumoniae, A	0.4	400^{24}
Salmonella typhi	25	400
Salmonella paratyphi B	200	>1600
Shigella sonnei	100	100
Staphylococcus aureus, 1	100	400
Pneumococcus, type 1	25	100^{14}
Pneumococcus, type 3	12.5	800^{30}
Streptococcus hemolyticus	100	800
Streptococcus viridans	50	400^{26}
Enterococcus	200	$>1600^{12}$
Staphylococcus aureus, 2	200	$>1600^{36}$

*Superscripts indicate number of subcultures when less than 40. For the sensitivity of these strains to neomycin, see Table V.

well under similar circumstances at each of the transfers from the antibiotic-containing plates to plates containing antibiotic-free media. It was then readily shown that a chloramphenicol-dependent variant had emerged which grew best in the presence of a critical, narrow range of concentrations of this antibiotic. When the concentration of chloramphenicol was either increased or decreased progressively from this critical level, there was a steady decline in growth until complete inhibition occurred. Evidence was also obtained of "back-mutation" of this strain to variants which were as sensitive as the original parent strain. Details of the emergence, isolation, and properties of this dependent strain have been reported elsewhere.[1]

Growth of the Chloramphenicol-Dependent Strain in Various Antibiotics.—The following experiment was carried out in order to determine whether any of four other antibiotics would support the growth of the chloramphenicol-dependent variant in the absence of chloramphenicol.

The surface growth of the dependent strain was transferred from an agar plate containing 50 μg. of chloramphenicol per milliliter to broth containing 12.5 μg. per milliliter. Subcultures were made twice at forty-eight hour intervals in broth containing similar concentrations of the antibiotic. The final growth was used for agar pour plates and colony counts in agar containing serial twofold concentrations of chloramphenicol, aureomycin, terramycin, streptomycin, and penicillin. Parallel pour plate cultures were also made with the parent strain which had been maintained without any antibiotics.

TABLE VIII. EFFECT OF VARIOUS ANTIBIOTICS ON THE GROWTH OF A CHLORAMPHENICOL DEPENDENT VARIANT OF KLEBSIELLA PNEUMONIAE

CONCENTRATION OF ANTIBIOTIC μG/ML.	NUMBER OF COLONIES PER MILLILITER OF A 10^{-4} DILUTION OF CULTURE*										CULTURE CONTROLS NO ANTIBIOTIC		
	CHLORAMPHENICOL		AUREOMYCIN		TERRAMYCIN		STREPTOMYCIN		PENICILLIN		DILUTION OF CULTURE	COLONIES PER ML.	
	C†	D	C	D	C	D	C	D	C	D		C	D
200	0	0	0	0	0	0	–	–	0	0			
100	0	0	0	0	0	0	–	–	0	0			
50	0	360	26	0	0	0	0	0	24	0	10^{-2}	++	0
25	0	404‡	37	0	0	0	0	0	53	0	10^{-3}	380	0
12.5	37	426	28	0	69	0	0	0	48	0	10^{-4}	40	0
6.3	42	0§	37	0	59	0	0	0	74	0	10^{-4}	34	0
3.1	43	0	38	0	56	0	0	0	55	0	10^{-4}	40	0
1.6	54	0	34	0	62	0	0	0	63	0			
0.8	41	0	29	0	62	0	0	0	53	0			
0.4	44	0	42	0	67	0	0	0	68	0			
0.2	49	0	28	0	39	0	55	0	–	–			
0.1	–	–	–	–	–	–	67	0	–	–			

*After incubation at 37° C. for forty-eight hours.

†C = Control: parent strain which had been maintained by serial transfers on plain agar; D = chloramphenicol-dependent variant.

‡Thirty-two of these colonies were picked, emulsified, and each suspension streaked on the surface of a plate containing 25 μg/ml. of chloramphenicol and on antibiotic-free agar. Growth in each instance occurred only on the former.

§One-hundred minute colonies were observed after incubation for seventy-two hours.

The results are shown in Table VIII. Visible colonies of the chloramphenicol-dependent variant developed after incubation for forty-eight hours in the presence of 50, 25, and 12.5 μg. of chloramphenicol per milliliter, and not in any other concentration of this agent, nor in the absence of antibiotic nor in the presence of any of the amounts of the other antibiotics used. The parent strain was completely inhibited by a limiting concentration of each of the five

antibiotics; no colonies developed during this period of incubation in greater concentrations, and essentially the same number of colonies developed in all of the lower concentrations. Thirty-two colonies of the dependent strain picked from the plate containing 25 μg. of chloramphenicol per milliliter all grew well when transplanted on the surface of agar containing the same concentration of this agent, but failed to grow on antibiotic-free agar.

*Identity of the Strains**.—Although considerable care was taken to avoid contaminations during the course of this study, the nature of the technical details and the fact that all of the cultures were used at the same time during each of the transfers offered possibilities for unavoidable errors. As already noted, control cultures of each of the five series of transfers were made each time on antibiotic-free media, and the surface growth was carefully inspected for changes in colonial morphology. Possible contaminants were examined and identified, and segments of culture plates containing suspicious contaminants were not used for the transfers. On several such occasions resort was made to previous subcultures to continue the series, and sometimes the entire series was discarded and a fresh start was made from the control strains. During the middle and after the end of the study, as indicated in the figures, the control cultures which had not been exposed to antibiotics were retested for sensitivity to all of the antibiotics.

In addition, the control strains and the corresponding ones from each of the four antibiotic series were studied for their serologic and/or biochemical reactions in order to get further information concerning their identity, and also to determine whether any changes might have resulted from the repeated exposures to the antibiotics. The corresponding strains from the five series were thus tested simultaneously at the end of the study. The results may be summarized briefly for each of the organisms.

E. coli.—All five cultures had identical colonial and microscopic morphology and gave identical biochemical reactions. The latter included fermentation of dextrose, lactose, and mannitol with production of acid and gas, failure to ferment sucrose, positive methyl red, and negative Voges-Proskauer tests.

A. aerogenes.—All five cultures appeared to be identical morphologically and in their biochemical reactions. All fermented dextrose, lactose, sucrose, and mannitol, with formation of acid and gas, gave negative indol and methyl red and positive Voges-Proskauer tests. The colonies were only slightly mucoid.

Klebsiella pneumoniae.—All five strains retained their mucoid colonial character and were specifically agglutinated by type A Friedlander's bacillus antiserum (rabbit), but all had lost their ability to produce capsular swelling with this serum. There were also differences in biochemical reactions: the control strain and the one which had been transferred forty times on aureomycin gave biochemical reactions identical with those of the strains of *A. aerogenes*. The chloramphenicol strain gave the same sugar reactions but positive indol and methyl red, but negative Voges-Proskauer tests. The terramycin and neomycin strains produced acid but not gas with the four sugars, was indol and V.P. negative, and methyl red positive.

*We are indebted to Miss Marion E. Lamb for the studies on the coliform and enteric bacilli, to Miss Clare Wilcox for identification of the strains of pneumococci and *H. influenzae*, and to Dr. E. L. Updyke of the Streptococcal Laboratory of the Communicable Disease Center for the classification of the streptococci.

S. typhi, S. paratyphi B and Sh. sonnei, Duval.—The five strains of each of these organisms gave identical and characteristic fermentations for the corresponding species and specific agglutination only with homologous antiserums.

Staphylococcus aureus, 1 and 2.—The strains from each of the five series of these two organisms were all hemolytic, produced coagulase, and had identical colonial morphology.

Pneumococcus, type 1 and type 3.—Each of the five cultures of both of these strains had identical colonial and microscopic morphology, and showed specific capsular swelling only with the homologous rabbit antipneumococcal typing serum.

Str. hemolyticus.—Four of the five strains reacted specifically with group A antiserum, but the aureomycin strain reacted with group B. Of the four group A strains, three were classified as type M30, but the control strain (no antibiotic exposures) failed to react with type 30 or with serums of any of thirty-three other available types.

Str. viridans.—All five grew in characteristic colonies on blood agar and gave identical biochemical reactions.

Enterococci.—All five strains resisted heating at 60° C. for twenty minutes, and reacted specifically with group D antiserum. Four were classified by their biochemical reactions as resembling *Str. zymogenes* and were beta hemolytic; the chloramphenicol strain resembled *Str. liquifaciens* and was less hemolytic.

Hemophilus influenzae.—All five strains had characteristic and similar colonial and microscopic morphology, but they all failed to react with capsular swelling when mixed with type-specific rabbit antiserums.

These results may be summarized briefly. Most of the strains either retained all of their original characteristics or showed parallel changes in the control strain and in the corresponding ones which had been transferred on the various antibiotics. The only significant changes in the same organisms among strains of the different series occurred in *Kl. pneumoniae* which showed diverse biochemical reactions, in one of the enterococci which also showed some biochemical variation, and in two of the strains of the hemolytic streptococcus which showed loss or changes in their serologic reactions.

DISCUSSION

In the present study a variety of bacteria was employed which was representative of the common aerobic human pathogens. Without discussing their relative merits, certain details of the methods used were different from those employed by others, and should therefore be mentioned. A considerably larger number of subcultures in the various antibiotics was employed in an attempt to attain more definitive results. The serial transfers were made from plates to plates after incubation for forty-eight hours, and rather large inocula were thus employed. The tests for sensitivity were done by the plate-dilution method; this was best adapted to the purpose at hand, but the values obtained with some antibiotics by this method may vary appreciably from those obtained by other methods.[2] Moreover, the end points of inhibition in these tests were sometimes not very sharp, and partial inhibition was not infrequently noted over a wide range of concentrations. Also, as mentioned earlier, growth of many strains could frequently be obtained by subculture from media containing much greater concentrations than those on which there was no discernible growth and which were considered to be the minimum complete inhibiting concentrations, as defined in this study. For that reason the maximum concentrations permitting

good growth have also been shown in the charts. The validity of the results, however, is confirmed by the fact that the latter values closely paralleled those of the minimum complete inhibiting concentrations.

The results of the present study suggest that the ability of an organism to develop resistance may depend on the antibiotic used. This is well recognized, but it is here shown, in addition, that with some antibiotics there may be considerable variation in the ability of different bacterial species (or even strains of the same species) to acquire resistance, and in the degree of resistance they may acquire as a result of repeated exposures. Thus, all of the organisms readily developed resistance to neomycin, and this usually occurred within a relatively small number of subcultures. The same was true, though not so strikingly or so rapidly with terramycin. On the other hand, some of the strains used here either increased only slightly or failed to increase in resistance to aureomycin and/or chloramphenicol during 40 consecutive subcultures. Furthermore, while some strains failed to develop resistance to both of these agents, others increased in resistance to one and not to the other. The strains that were initially the most sensitive usually were among those which failed to acquire resistance but this was not always the case and some strains of relatively low sensitivity (e.g., *A. aerogenes* and *Sh. sonnei*) failed to increase in resistance to aureomycin.

With respect to cross-resistance, there were also differences related to the antibiotics on the one hand and to the organisms on the other. Thus, of the four antibiotics studied here, resistance to neomycin was most specific, inasmuch as organisms which had become resistant as a result of exposures to that agent did not simultaneously increase in resistance to aureomycin, terramycin, and chloramphenicol. Conversely, strains that had acquired resistance to any of these three antibiotics did not increase in resistance to neomycin. The rather marked increase in resistance to streptomycin resulting from exposures to neomycin, however, was unexpected and is, therefore, of particular interest. This aspect is being explored further. Penicillin resistance did not develop in any of the organisms tested after exposures to any of the four antibiotics that were studied.

Aureomycin and terramycin showed the development of mutual cross-resistance most clearly and regularly. The results with chloramphenicol, on the other hand, varied with the species, some strains developed significant cross-resistance to terramycin and/or aureomycin while others showed little or none.

Several studies dealing with cross-resistance among antibiotics have been reported since the brief preliminary note of the present study was made.[3] Heilman[4] made strains of *E. coli*, *A. aerogenes*, and *Str. faecalis* resistant to aureomycin, terramycin, chloramphenicol, or streptomycin by serial subcultures in broth containing increasing amounts of the individual agents, and then tested their sensitivity to all of them. Strains made resistant to any one of the first three agents increased in resistance to all three but not to streptomycin. Conversely, strains made resistant to streptomycin did not increase in resistance to the other three antibiotics. As was the case with neomycin in the present

study, the exposures to streptomycin often resulted in increased sensitivity to the other agents and vice versa, some of the organisms made resistant to the latter became more sensitive to streptomycin.

Pansy and associates,[5] using a strain of *E. coli* and one of *Staph. aureus*, produced increased resistance to aureomycin or chloramphenicol by five to ten transfers in broth containing increasing concentrations of one of these antibiotics. Each organism then developed resistance, at somewhat lower levels, to the other agent and also to terramycin. Fusillo and Romansky[6] found that several strains of *A. aerogenes*, *E. coli*, and *Staph. aureus* recovered from patients after treatment with aureomycin were resistant to that agent and to terramycin as well. In one of these patients the organism initially was sensitive to both of these antibiotics. The latter authors also succeeded in increasing the resistance of a few originally sensitive strains of these organisms and of *Str. faecalis* to both aureomycin and terramycin by serial transfers (ten times in terramycin and an unspecified number of times in aureomycin) in broth containing either of these agents.

Kaipainen[7] studied cross-resistance in thirty-seven strains of nine species following seven transfers of each in broth containing aureomycin, chloramphenicol, terramycin, dihydrostreptomycin, and penicillin. He found that when strains of *E. coli*, *A. aerogenes*, *S. typhi*, *Pseudomonas aeruginosa*, or *Proteus vulgaris* were increased in resistance to either aureomycin, terramycin, or chloramphenicol, a simultaneous increase in resistance followed to the other two but the organisms at the same time became more sensitive to dihydrostreptomycin. Increases in resistance to dihydrostreptomycin resulting from the exposures to that agent were accompanied by increased sensitivity to the other three antibiotics. Variable results, however, were obtained with strains of *Staph. aureus*, *Staph. albus*, *Str. pyogenes*, and *B. subtilis*. Cultivation of any of the organisms in broth containg penicillin did not alter the resistance to any of the other four agents and, conversely, increases in resistance to any of those agents did not affect the sensitivity to penicillin.

Other studies involving cross-resistance among closely related agents, such as the various penicillins or streptomycins, or among different types of antibiotics, have been reviewed by some of the authors quoted.[5, 7] In general, the development of cross-resistance has been limited largely to chemically related antibiotics. Cross-resistance between streptomycin and neomycin has not been encountered,[8-10] but this has not been studied extensively. Except for chloramphenicol, the precise chemical structures of the antibiotics with which we have been chiefly concerned in the present study are not known. In the case of neomycin, moreover, the material used was not highly purified, and may have contained a mixture of related substances. As noted by Pansy and associates[5] it is reasonable to assume that cross-resistance may indicate interference with the same biologic processes. It is not possible from the present or any of the previous studies, however, to draw any reliable conclusions concerning the relationships of the chemical structures of these four antibiotics.

The problem of the identity of the strains was considered in detail because of the possible changes that may result from repeated subcultures of some bac-

teria on various media, both with or without antibiotics. Some of the discrepancies that were observed may, of course, have been the result of unrecognized contamination during the course of the serial transfers, but the possibility of mutations has not been excluded, and probably accounts for most of the changes noted. The irregular values obtained in some of the sensitivity tests may have been the result of such mutations or contaminants.

The chloramphenicol-dependent mutant was of special interest. As pointed out elsewhere,[1] the methods used did not preclude the possibility that similar variants had developed in other organisms or with other antibiotics and were not recognized. The fact that none of four other antibiotics, in a wide range of concentrations, permitted growth of the chloramphenicol-dependent variant in the absence of chloramphenicol suggests that the structural component of the latter agent which is necessary to permit multiplication of this variant was not available in utilizable form in any of the other four antibiotics which were tested.

SUMMARY AND CONCLUSIONS

Parallel serial transfers were made with fourteen bacterial strains on solid media containing progressively increasing concentrations of aureomycin, terramycin, chloramphenicol, and neomycin, and on media containing no antibiotics. Forty such transfers were made in most instances, and the final subculture of each series was tested for sensitivity to all four antibiotics. Some of the strains were also tested for sensitivity to penicillin and the organisms which had been exposed to neomycin were also tested with streptomycin.

The development of increased resistance to the homologous and heterologous antibiotics varied with the antibiotics and with the strains which had been exposed to them.

Resistance to the homologous antibiotic increased most regularly and most rapidly to neomycin; this also occurred regularly, though not so strikingly with terramycin. Increased resistance to aureomycin and chloramphenicol occurred in some strains but not in others following their exposure to these respective agents.

Repeated exposures to aureomycin or terramycin resulted in the development of mutual cross-resistance to these agents most regularly and clearly. The results with chloramphenicol varied with the organism—following exposures to that agent some strains developed significantly increased resistance to aureomycin and/or terramycin, while others did not. Conversely, some strains rendered resistant to either aureomycin or terramycin also developed significantly increased resistance to chloramphenicol, while such was not the case with other strains.

Organisms rendered resistant to neomycin as a result of repeated subcultures on this antibiotic showed no increase in resistance to aureomycin, terramycin, or chloramphenicol, and in some instance appeared to become more sensitive to these three agents. Conversely, strains which had increased in resistance to aureomycin, terramycin, or chloramphenicol generally showed unchanged

sensitivity to neomycin. Almost all of the strains which had increased in resistance to neomycin, however, also increased significantly and sometimes markedly in resistance to streptomycin.

None of the strains tested had changed in their sensitivity to penicillin as a result of repeated exposures to either aureomycin, terramycin, chloramphenicol, or neomycin.

A chloramphenicol-dependent variant of *Klebsiella pneumoniae*, which emerged in the course of this study, grew only in the presence of a narrow critical range of concentrations of chloramphenicol but not in greater nor smaller concentrations of that agent, nor in the presence of any of a wide range of concentrations of four other antibiotics.

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THE MIDDLEBROOK HEMAGGLUTINATION TEST FOR TUBERCULOSIS

An Evaluation in Normal Subjects and in Patients With Tuberculosis

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In 1948 Middlebrook and Dubos[1] described the specific serum agglutination of erythrocytes which had been sensitized with an extract of tubercle bacilli. Using this technique it was possible to demonstrate circulating antibody in rabbits that had received BCG and in patients with active tuberculosis. On the basis of the limited studies reported at that time the test was regarded as being highly specific. The material responsible for the reaction was considered to be a polysaccharide, and it was noted in the original article that one particular old tuberculin preparation was as satisfactory as was the specially prepared extract of tubercle bacilli.

Following this, two groups of investigators[2-4] reported that another preparation of old tuberculin* is also satisfactory and yields highly specific results. This work has been accepted by Middlebrook who states in an editorial[5] that a positive reaction at 1:8 or higher dilution of serum is presumptive evidence of disease due to tubercle bacilli or to a *Mycobacterium* of closely related antigenic structure. These conclusions have, of course, stimulated considerable interest in this subject. The studies here reported represent a part of a larger program set up to follow the behavior of the hemagglutination test in a sizable group of medical students and student nurses receiving BCG vaccination.

MATERIALS AND METHODS

Serum specimens were obtained, just prior to tuberculin testing, from the entering freshman medical students and student nurses at the University of Michigan in the fall of 1950. A chest roentgenogram was done on each subject. There was a total of 264 such subjects. Of these eight are excluded from present consideration by reason of roentgenographic evidence of clinical tuberculosis, or a history of tuberculosis or previous BCG vaccination, leaving 256 normal apparently healthy young adults. Among the 256 there were 55 who gave a positive reaction to the intradermal injection of 0.0001 mg. P.P.D. For purposes of comparison, the sera from 66 patients† with moderately or far-advanced, active, pulmonary tuberculosis were included in the study. Serum specimens were inactivated by heating at 56° C. for thirty minutes, and were then frozen and stored in the frozen state until tested.

The Use of Human Type "O" Erythrocytes.—The use of sensitized sheep red cells necessitates prior absorption of the serum with unsensitized sheep cells to remove naturally occurring heterophile antibodies. This procedure is time consuming and involves the use of large quantities of expensive cells. In these studies human type "O" red cells from a single

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Supported by a grant from the Research and Education Service, Department of Medicine and Surgery, Veterans Administration.
Received for publication, July 9, 1951.

*Lederle 4 times standard strength tuberculin. This same preparation was supplied for the present studies through the courtesy of Dr. H. D. Piersma.

†We are indebted to Dr. W. F. Fidler of the Michigan State Sanatorium, Howell, Michigan, for many of these sera.

TABLE I. SOURCE OF SERUM SPECIMENS FOR COMPARING THE UTILITY OF SENSITIZED SHEEP ERYTHROCYTES AND HUMAN TYPE "O" ERYTHROCYTES

SPECIMEN NUMBER	DESCRIPTION OF DONOR
1	Normal adult—B.C.G. vaccination one year previously.
2	Minimal pulmonary tuberculosis—active.
3	Normal adult—tuberculin positive.
4	Normal adult—B.C.G.—vaccination one year previously.
5	Pulmonary tuberculosis—advanced—probably arrested.
6	Normal adult—tuberculin negative.
7	Pulmonary tuberculosis—advanced—active.
8	Normal adult—B.C.G. vaccination one year previously.
9	Chronic ulcerative colitis—reversal of A/G ratio.
10	Lymphoblastoma—arrested pulmonary tuberculosis.

source were used. The use of these cells was justified by the following experiment: large amounts of serum were collected from ten subjects representative of various patterns of hemagglutination. The pertinent data on each of these subjects is given in Table I. Aliquots from each specimen were absorbed, according to the original technique,[1] with sheep cells from two different sheep and with guinea pig kidney.[6] Each of the thirty specimens thus obtained was then titered against sensitized human "O" cells and against sensitized sheep cells from each of the two sheep. The results are shown in Table II. The titers obtained with the use of human "O" cells were the most consistent, although they were generally slightly lower than those obtained with sheep cells. The greatest variation was noted when serum absorbed with cells from one sheep was run against sensitized cells obtained from the second sheep. In one instance (serum 10) absorption with cells from one sheep failed to remove antibody capable of agglutinating unsensitized cells from the other source, as evidenced by a positive control. Guinea pig kidney, as expected, was unsatisfactory since it does not remove the heterophile antibody acquired following infectious mononucleosis. It is of some interest that agglutination in the control tube (serum plus unsensitized sheep cells) occurred in seven of the ten sera after absorption with guinea pig kidney. Further justification for the use of human red cells was obtained by duplicate testing, from time to time, of groups of sera employing both human cells and sheep cells. There was usually no more than one tube variation in titers.

Sensitization of Erythrocytes.—Human type "O" red cells were stored at 5° C. in sterile Alsever's solution until used. Old tuberculin of four times standard strength

TABLE II. COMPARISON OF HEMAGGLUTINATION TITERS OBTAINED USING SHEEP CELLS FROM TWO SOURCES AND HUMAN TYPE "O" CELLS

	ABSORPTION OF SERA								
	SHEEP A RED CELLS			SHEEP B RED CELLS			GUINEA PIG KIDNEY		
	SOURCE OF SENSITIZED CELLS								
SERUM NO.	SHEEP A	SHEEP B	HUMAN TYPE "O"	SHEEP A	SHEEP B	HUMAN TYPE "O"	SHEEP A	SHEEP B	HUMAN TYPE "O"
1	128*	32	16	16	32	8	32	64	8
2	16	4	4	2	16	4	16†	16†	2
3	0	0	0	0	0	0	2†	4†	0
4	32	64	16	8	32	16	64	64†	16
5	2	2	0	4	8	2	8	8†	2
6	8	8	2	0	4	0	4	16	0
7	128	128	64	64	128	64	128	64	64
8	128	128	8	16	64	16	64†	64†	8
9	0	4	0	0	0	0	2	4†	0
10	0	16†	0	0	2	0	4†	8†	0

*Numbers represent dilutions; i.e., 128 means 1:128.

†Positive control; serum agglutinated unsensitized cells in 1:2 dilution.

In using sensitized human "O" cells, the prior absorption of sera is unnecessary. In the above experiment, absorbed sera were used throughout in order to compare specimens handled in the same fashion and to nullify any minor degree of dilution difference which may have occurred in the process of absorption.

(Lederle) was diluted 1:12 by addition of 1 ml. to 11 ml. of buffered isotonic saline. To this was added 0.25 ml. of packed washed red cells, and the mixture was incubated for two hours at 37° C. with thorough shaking at fifteen-minute intervals. The cells were then washed with buffered saline three times and were suspended in 50 ml. of buffered saline. This suspension of sensitized cells was stored at 5° C., and any unused portion was discarded after three days.

Performance of the Test.—Serum diluted 1:2 was added in 0.5 ml. quantity to each of the first two of a series of standard Kahn tubes. Serial twofold dilutions were made from the second tube through seven or more tubes using equal volumes of buffered saline. To each tube was added 0.5 ml. of the sensitized cell suspension. The racks of tubes were then incubated for two hours at 37° C., were thoroughly shaken, and allowed to stand overnight at room temperature when they were read. Clear-cut evidence of macroscopic agglutination was considered a positive reaction. Control tubes containing serum in 1:2 dilution plus unsensitized cells were added for each specimen. A positive control was never encountered when human "O" cells were used. Known positive and negative sera were included in each day's run. Tests were reproducible within the limits of one tube variation.

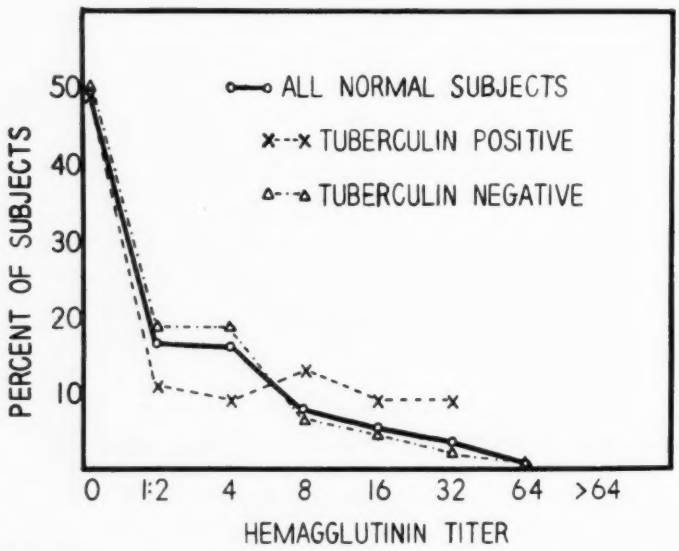

Fig. 1.—The distribution of hemagglutination titers in 256 normal young adults. The distribution curves for the 55 tuberculin positive and 201 tuberculin negative members of the group are also illustrated.

RESULTS

The hemagglutination titers in the sera from the 256 normal young adults ranged from 0 to 1:64, and in the 66 patients having moderately or far advanced, active, pulmonary tuberculosis the range was from 0 to 1:128. Fig. 1 shows the range and distribution of titers in the normal group, and in the tuberculin-positive and tuberculin-negative members of this group. While the range of titers was quite similar for normal subjects and patients, there was considerable variation in the distribution of the various titers, as is illustrated in Fig. 2. Among the patients with pulmonary tuberculosis there was a lower incidence of negative reactions (16.6 per cent as compared to 49.6 per cent in normal subjects) and the peak incidence occurred at the 1:16 level. The

TABLE III. COMPARATIVE DATA ON HEMAGGLUTINATION TESTS OF SERA FROM NORMAL SUBJECTS AND PATIENTS WITH ADVANCED, ACTIVE, PULMONARY TUBERCLUOSIS

DIAGNOSIS	TOTAL	NO REACTION		TITERS 1:8 OR GREATER		MEAN TITER*
	NO.	NO.	%	NO.	%	
Normal subjects	256	127	49.6	44	17.1	3.8
Tuberculin +	55	27	49.0	17	30.9	5.9
Tuberculin −	201	100	49.7	27	13.4	3.2
Tuberculosis patients	66	11	16.6	36	54.5	12.3

*If it is postulated that it requires one unit of agglutinating substance to give a positive reaction, then a serum agglutinating in dilution of 1:64 contains 64 such units. The mean titer, for a given group of sera, is arrived at by totaling the number of such units in the group and dividing by the number of specimens.

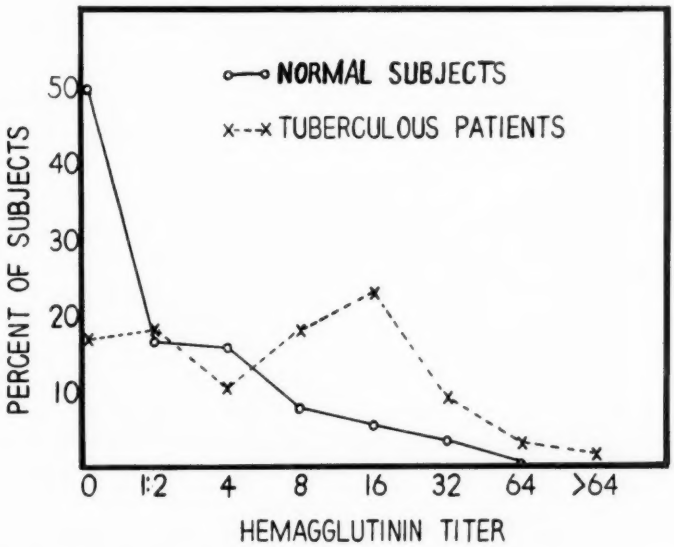

Fig. 2.—The distribution of hemagglutination titers for 256 normal subjects and for 66 patients with moderately or far advanced pulmonary tuberculosis.

mean titer, which is one method of expressing the average amount of antibody within a given group of sera, was significantly higher in the tuberculosis patients.

It has been stated[3, 5] that a positive reaction in dilution of 1:8 or above is indicative of active tuberculosis. In this study, titers of this magnitude were found to be present in 17.1 per cent of the sera from the group of normal young adults in whom the existence of tuberculosis had been excluded by all practical means, and 54.5 per cent of the patients with active pulmonary tuberculosis. These data are summarized in Table III.

DISCUSSION

In the studies here reported the range of titers in the sera from normal subjects was virtually identical with those found in the sera from patients with advanced, active, pulmonary tuberculosis. Other investigators[3, 5] have suggested that a positive reaction in dilution of 1:8 or above is of significance.

If this criterion is applied to the present data, significant reactions occurred in only 54 per cent of patients with active tuberculosis, and the incidence of what must be regarded as false positive reactions was 17 per cent. Under these circumstances it is very doubtful that the test, as it now stands, is at all reliable in the diagnosis of tuberculosis or as an index of activity in patients known to have the disease.

Limited observations on the sera from patients with a variety of diseases have so far yielded no information that would help to explain the false positive reactions. The elimination of these reactions by improvement of the antigen, or through the identification and selective removal of the responsible antibody, could conceivably result in a test of high specificity.

Since some degree of agglutination occurred in 50 per cent of the sera from normal subjects, the possibility of some type of receptor change in the red cells must be considered. Certain alterations of this kind have long been known to render red cells pan-agglutinable. Thomsen,[7] in 1926, observed that red cells taken from contaminated blood specimens were often agglutinated by normal serum of homologous type. Friedenreich[8] later identified one cause of this phenomenon as a heat labile substance present in the filtrate from cultures of certain diphtheroids. Since that time it has been shown that a similar alteration of red-cell receptors can be produced by numerous bacterial metabolites,[9] by other enzymes,[10, 11] and even by simple inorganic substances such as periodate.[12, 13] Once the red cells have been altered in this fashion, they are agglutinable by various percentages of normal sera. Thus it is possible that the treatment of red cells with old tuberculin, a heterogeneous mixture of numerous substances, could result in alterations which would render the cells agglutinable by normal sera. While the majority of the work on such pan-agglutination has been done on human erythrocytes, it is apparent from the work of Friedenreich[8] that sheep cells are also subject to this phenomenon. The use of human type "O" erythrocytes simplifies the Middlebrook hemagglutination test by eliminating the need for absorption of heterophile antibodies.

The antibody responsible for pan-agglutination (called "T-agglutinin" by Friedenreich) may be removed from sera by absorption with treated red cells, and this offers a possible approach toward increasing the specificity of the hemagglutination test for tuberculosis.

The results, herein reported, of studies on the hemagglutination test for tuberculosis are in virtually complete agreement with those of two other investigations which have been recently reported.[14, 15]

SUMMARY AND CONCLUSIONS

The hemagglutination test for tuberculosis was applied to the sera obtained from a group of 256 normal young adults and 66 patients with moderately or far-advanced active pulmonary tuberculosis.

Positive reactions in dilutions of serum of 1:8 or greater were found in 44 (17.1 per cent) of the normals, and in 36 (54.5 per cent) of the patients with tuberculosis.

It is doubtful that the test, as it now stands, is of any value as a diagnostic procedure or as an index of activity of disease.

Human type "O" red cells are as satisfactory as sheep cells in the performance of the test, and the use of these cells is saving of time and expense.

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FATAL HERPES SIMPLEX INFECTION IN A NEWBORN INFANT

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ALTHOUGH there is little information in regard to herpes simplex infection in the newborn infant, the fundamental observations concerning the relationship between man and this virus have been clearly pointed out. It has become increasingly apparent that the initial infection frequently manifests itself as a form of gingivostomatitis in children between the ages of 1 and 3 years,[1-5] although older children and adults have also been shown to have this primary form of the disease. The initial infection, having an incubation period of three to four days, usually does not last beyond two weeks. However, it has been suggested that a child infected in this manner continues to harbor the virus for the remainder of his life, and a fairly good equilibrium between virus and host exists, the presence of the former becoming manifest from time to time in the form of herpes labialis. The association of recurrent cold sores and illness, trauma, minor emotional upsets, etc., are well known facts. Such a pattern can also be explained as being due to repeated episodes of newly introduced infection.[6]

Occasional individuals are subject to recurrent episodes of herpetic gingivostomatitis,[7-9] and eczematous individuals tend to have a generalized herpetic involvement of the skin.[9-11] A further spread of the virus in man has been noted in those instances when it has penetrated the cells of the brain and meninges and caused encephalitis and meningitis.[12-14]

Its widespread distribution is well demonstrated by a sampling of an adult group for the presence of neutralizing antibody. From 30 to 90 per cent of different groups of adults show antibody against the herpes virus.[2, 15, 16] The range in figures appears to be related to socio-economic levels, the lower levels showing more individuals with evidence of experience with herpes virus.[2] The first appearance of antibody develops within two to four weeks after the episode of primary gingivostomatitis, although some individuals develop antibody without any manifest evidence of contact with the virus.

It is surprising that such a commonly encountered pathogen has not been associated more with illness in the newborn infant. It is hoped that attention may be focused on this interesting phase of herpes infection by reporting the apparent transmission from mother to newborn infant of what appeared to be

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Conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, Division of Preventive Medicine, Office of the Surgeon General, United States Army, Washington, D. C.

Received for publication, June 22, 1951.

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primary herpetic stomatitis in the mother and a generalized herpetic involvement in her newborn baby, with fatal termination as the result of probable encephalitis.

CASE REPORT

Illness in the Mother.—Mrs. M. B., aged 22 years, developed a small vesicular lesion on the inner surface of the upper lip nine days ante partum. In the following three days the lesions had progressed to involve the gums and tip of the tongue as well as the entire lower lip. At the time of birth a few lesions still persisted on the lower lip and the gums "were sore." At no time was she aware of genital lesions, nor were any noted at the time of delivery. No previous episodes of a similar nature had occurred, although she had had occasional "sores" in the mouth but not on the lips. The baby was first seen by her at three days of age, and four days after delivery she fed the baby at her breast.

Illness in the Newborn Infant.—B. B. was born nine days after the onset of his mother's stomatitis. The delivery and neonatal period were uneventful until the seventh postpartum day when some erythematous, vesicular lesions were first noted over the anterior thorax. At that time the appetite was good and a 75 Gm. weight gain was registered in the preceding twenty-four hour period. Despite the use of sulfadiazine ointment, the lesions became more numerous, and on the ninth day lethargy, respiratory distress, cyanosis, and convulsive movements of both arms and legs were noted. The temperature was 96° F., and later rose to 103° F. At that time the lesions on the trunk had become larger, and in some areas were coalescent with a central zone of whitish-gray necrotic material surrounded by a thin rim of erythematous skin. The vesicular lesions had become much larger, and the majority had reached a purulent stage. Analyses of blood and urine were normal except for some albumin in the latter. Examination of the spinal fluid indicated no abnormality. Treatment consisted of penicillin, sulfadiazine, blood transfusions, and gamma globulin. During the following seventy-two hours the encephalitic symptoms became more pronounced with severe convulsions, opisthotonos, and a fatal termination five days after the onset of clinical signs. Blood cultures on the second and last day of illness were negative, as was bacteriologic culture of the vesicle fluid. No lesions were noted in the mouth.

VIRUS STUDIES

Scrapings from the newborn infant's skin lesions were obtained on the second day of illness when he was seen for the first time. Later, postmortem sections of liver were also obtained and showed not only numerous macroscopic lesions but also areas of focal necrosis with little cellular inflammatory reaction. The hepatic cells showed acidophilic intranuclear inclusions surrounded by a clear zone with margination of the chromatin, type A inclusions of Cowdry.[20] The antemortem skin scrapings were placed on the cornea of a rabbit's eye, and in four days a purulent keratitis developed. The nictating membrane was removed and triturated with alundum,[16] then made up to approximately a 10 per cent suspension with 10 per cent horse serum in saline. The supernatant fluid was injected intracerebrally in mice, and in the third mouse passage was causing encephalitis and death in three to five days. Sections of the brains showed type A inclusions in the cell nuclei.

Virus was also obtained from the specimens of postmortem skin and liver from the infant. All three specimens were concomitantly adapted to the chorioallantoic membranes of chick embryos.[17] All passage material was cultured for bacterial contamination; if any was noted such specimens were discarded. It is worth noting that penicillin and streptomycin were added to

the original specimens before being injected into animals or eggs, although both antemortem and postmortem specimens were subsequently found to be bacteriologically negative.

After adaptation of the virus obtained from the antemortem skin scrapings to the chorioallantoic membrane, pock neutralization tests[18] were done using the normal and convalescent sera from two rabbits which had been immunized against the Armstrong strain of herpes simplex[13] virus and another strain of herpes simplex virus (Hajas), isolated in this laboratory from an individual with recurrent labial lesions. The results listed in Table I show that sera from rabbits immune to known strains of the herpes simplex virus neutralized the BB virus.

TABLE I. EGG NEUTRALIZATION TESTS WITH THE BB VIRUS

HERPES IMMUNE RABBIT SERA	SERUM STAGE	TITER OF POCK NEUTRALIZING ANTIBODY
Armstrong strain	normal	1:2
	immune	1:16
Hajas strain	normal	1:2
	immune	1:32

10 ID_{50} of BB virus was mixed with each dilution of serum.

Other, more complete cross-neutralization tests were done in 11-day-old chicken embryos.[19] The results are shown in Table II, and demonstrate the close immunologic relationship of the BB strain to HF, another well-established herpes strain (obtained from Dr. I. Ruchman, University of Cincinnati, College of Medicine, Department of Pediatrics), and a strain of herpes simplex virus isolated from a 13-month-old infant with primary herpetic stomatitis (Prit.).

TABLE II. CROSS-NEUTRALIZATION TEST IN EMBRYONATED EGGS OF STRAINS OF HERPES SIMPLEX VIRUS AND SPECIFIED IMMUNE RABBIT SERA. RESULTS EXPRESSED AS ID_{50}

RABBIT SERA	BB VIRUS	PRIT. VIRUS	HF VIRUS
Normal	$10^{-4.3}$	$10^{-4.3}$	$10^{-4.5}$
BB Immune	$10^{-1.5}$	$10^{-2.5}$	$10^{-1.5}$
Prit. Immune	$10^{-1.7}$	$10^{-1.5}$	$10^{-1.5}$

Egg pock neutralization tests using sera obtained from the mother four weeks and six weeks after her episode of stomatitis showed antibodies in both specimens when tested against the virus isolated from her newborn infant.

The isolation of herpes simplex virus from the skin and liver of this newborn infant plus the history of a possible herpetic infection in the mother strongly suggests an intimate relationship between the two illnesses. The early course of the illness in the newborn infant was similar to impetigo, and later sepsis was seriously considered. However, the negative evidence from culture of the vesicle fluid and blood by anaerobic and aerobic means seemed to rule out most bacterial causes. Type A acidophilic inclusion bodies[20] in the hepatic cells further strengthened the probability of a virus etiology.

The finding of skin and liver lesions plus the probability of encephalitis would indicate a generalized infection in the newborn. Furthermore, the ab-

sence of lesions in the mouth mediates against the oropharynx as the portal of entry. The presence of skin lesions suggests entry through this organ with subsequent dissemination through the newborn infant.

DISCUSSION

The occurrence of such a sequence of events in newborn infants has some support from experimental infections in mice,[21] where it was shown that newborn mice were susceptible to intranasal herpes simplex infection. However, it was also shown that mice born of herpes immune mothers were more resistant. This information suggests that infection of a newborn infant would not be as severe if the mother had had previous experience with and antibody to the herpes simplex virus. In the case described, the presumption of a primary gingivostomatitis in the mother seems most likely, and a subsequent transmission to the baby, where a lack of antibody allowed widespread dissemination of the virus infection. In any event, the occurrence of a primary herpetic infection in an expectant mother should be a warning signal and focus attention on the possibility of a generalized dissemination of the virus in the newborn infant.

CONCLUSIONS

A strain of herpes simplex virus was isolated from antemortem skin and postmortem skin and liver in a newborn infant who showed evidence of vesicular lesions of the skin plus probable encephalitis. Histologically areas of focal necrosis of the liver with intranuclear inclusions of the hepatic cells were also found.

It was assumed that an episode of vesicular gingivostomatitis in the above infant's mother occurring in the immediate antepartum period was caused by a strain of herpes simplex virus, and represented the source of infection in the newborn.

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SIMULTANEOUS RECOVERY OF TYPE A′ AND TYPE C INFLUENZA VIRUSES FROM A PATIENT

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SIMULTANEOUS infection in human beings with type A-A′ and type B influenza viruses has been observed infrequently. However, in one instance, a high incidence of concurrent infection with type A′ and type C influenza viruses in children has been reported.[1] In all these instances the evidence of a dual infection was a rise in the level of serum antibodies against both types of virus. The isolation of two types of influenza virus from a patient has not been recorded previously. The rarity of dual infections and dual isolations can be attributed in part to the fact that the infection with a single type of virus tends to predominate in any influenza epidemic. In addition, it is possible that the interference phenomenon may play a role in minimizing the likelihood of dual infections in human beings and in eggs. The simultaneous isolation of type A′ and type C influenza virus from a patient seemed of sufficient interest to warrant reporting in detail.

MATERIALS AND METHODS

In February, 1951, during an epidemic of influenza A′ at Ann Arbor, Michigan, a patient, B. P., with symptoms of mild influenza, was admitted to the student health service infirmary. A summary of the clinical record of this patient follows:

CASE 1.—B. P., a 20-year-old white student nurse, was hospitalized Feb. 18, 1951, with chief complaints of chills, feverishness, and muscle aches of twenty-four hours' duration. Past history was noncontributory. She had not been vaccinated against influenza. On February 17, the patient noted the abrupt onset of malaise, lassitude, chilliness, and alternately a sensation of feverishness, which frequently was terminated by profuse diaphoresis. Muscle aches in the region of the thighs, back, and shoulder girdle and a dull retro-orbital aching sensation were noted. A mild sore throat, a nonproductive cough, and hoarseness appeared shortly after the onset of the above symptoms.

On admission the patient's rectal temperature was 101.5° F., radial pulse 100, and respirations 22 per minute. The nasal mucous membranes appeared inflamed, and the pharynx was moderately injected. Palpable cervical lymph adenopathy was recorded. The chest was clear to percussion and auscultation. The rest of the physical examination was within normal limits. The blood count on admission was: hemoglobin 15.3 Gm. per cent, white blood cells 4,650 per cubic millimeter, polymorphonuclears 76 per cent, lymphocytes 21 per cent, monocytes 2 per cent, and eosinophils 1 per cent.

The patient was afebrile by February 20, and was asymptomatic upon discharge, February 21, save for easy fatigability and lassitude which persisted for the following week. This patient was considered to represent a mild attack of influenza which was clinically indistinguishable from the other cases of influenza seen during this epidemic.

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This investigation was conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D. C.

Received for publication, June 30, 1951.

A throat washing was obtained on the third day of this patient's illness and was stored at 4° C. overnight. The next day 2 ml. were removed and 1,250 units of penicillin and 0.25 mg. of streptomycin were added in a volume of 0.1 ml. The mixture was inoculated intra-amniotically into eight ten-day embryonate eggs in 0.1 ml. amounts. After incubation at 35° C. for three days, the amniotic fluid was harvested from each of the six surviving eggs. The fluids were diluted tenfold in 2 ml. of physiological saline. These dilutions were then distributed in 0.5 ml. quantities to four separate tubes. To two tubes of each dilution 0.5 ml. of 0.5 per cent chicken erythrocytes was added, and to the remaining two tubes was added 0.5 ml. of 0.5 per cent guinea pig erythrocytes. One set of tubes containing cells of each species was incubated at room temperature, while the second set was incubated at 6° C. The hemagglutination patterns at the end of one and one-half hours are recorded in Table I.

TABLE I. HEMAGGLUTINATION REACTIONS OF FLUIDS FROM FIRST EGG PASSAGE OF B. P. THROAT WASHING

PASSAGE NO.	AMNIOTIC FLUID NO.	INCUBATION AT 25° C.		INCUBATION AT 6° C.	
		CHICKEN R.B.C.	GUINEA PIG R.B.C.	CHICKEN R.B.C.	GUINEA PIG R.B.C.
1 (Original throat washing)	1	0	0	+	0
	2	0	0	+	0
	3	0	0	0	0
	4	0	+	+	+
	5	0	0	+	0
	6	0	0	+	0

RESULTS

It will be noted that fluids No. 1, 2, 5, and 6 agglutinated chicken erythrocytes at icebox but not at room temperature, while guinea pig erythrocytes were not agglutinated at either temperature. In contrast, fluid No. 4 agglutinated chicken red blood cells in the cold, and, in addition, caused the agglutination of guinea pig erythrocytes at both room and icebox temperatures. Since influenza type C virus will agglutinate chicken red blood cells at 6° C. more readily than at 25° C., but will not agglutinate guinea pig red blood cells at either temperature,[2] and since recently isolated strains of other types of influenza virus may agglutinate guinea pig red blood cells more readily than chicken erythrocytes,[3] it seemed likely that these fluids contained both type C and type A′ influenza virus either separately or in mixtures.

To test this hypothesis, the following experiments were carried out: fluids No. 1, 2, 5, and 6 were pooled for passage and diluted one hundred fold in saline containing 10 per cent normal horse serum. A group of eight ten-day embryonate eggs was inoculated intra-amniotically with this material. At the same time another group of eight eggs was inoculated with the same material containing type A′ immune ferret serum in a final dilution of 1 to 10. Fluid No. 4 was similarly diluted and passed to six eggs intra-amniotically and four eggs intra-allantoically without the addition of immune serum. The hemagglutination reactions of the fluids harvested from these passages are recorded in Table II. Amniotic fluids were tested individually. The allantoic fluids from the four eggs inoculated intra-allantoically were pooled prior to testing.

It can be seen that, on the basis of the hemagglutination reaction with each species of cells, the passage of the pooled fluids made in the absence of

TABLE II. HEMAGGLUTINATION REACTIONS OF FLUIDS FROM SECOND EGG PASSAGES OF B. P. THROAT WASHING

PASSAGE NO.	AMNIOTIC FLUID NO.	INCUBATION AT 25° C.		INCUBATION AT 6° C.	
		CHICKEN R.B.C.	GUINEA PIG R.B.C.	CHICKEN R.B.C.	GUINEA PIG R.B.C.
	1	0	0	+	0
2	2	0	0	+	0
	3	0	0	+	0
Pool of No. 1, 2, 5, 6 fluids from passage 1 without immune serum	4	+	+	+	+
	5	0	+	+	+
	6	0	0	+	0
	7	0	0	+	0
	8	+	+	+	+
	1	0	0	+	0
2	2	+	0	+	0
	3	+	0	+	0
Same pool as above with Type A′ immune ferret serum	4	+	0	+	0
	5	+	0	+	0
	6	+	0	+	0
	7	0	0	+	0
	8	+	0	+	0
	1	0	+	+	+
2	2	0	+	+	+
	3	+	+	+	+
No. 4 fluid from passage 1 passed intra-amniotically	4	+	+	+	+
	5	+	+	+	+
	6	+	+	+	+
2 No. 4 fluid from passage 1 passed intra-allantoically	Pooled All. Flds.	+	+	+	+

type A′ immune serum contained a mixture of type A′ and type C influenza virus, while the passage made in the presence of type A′ immune serum contained type C virus alone. The hemagglutination reaction of the intra-amniotic and the intra-allantoic passages of fluid No. 4 is consistent with that shown by type A′ influenza virus, although the presence of type C influenza virus in these fluids cannot be excluded by this test.

TABLE III. IDENTIFICATION OF THE TWO VIRUSES ISOLATED AND OF THE DUAL INFECTION OF PATIENT B. P. BY THE H.I. TEST

SERUM	STAGE	RHODES TYPE A′	B.P. NO. 4 FLUID LINE	JJ TYPE C	B. P. POOLED FLUIDS LINE
Ferret 033	Normal	32*	64	32	32
	Type A′ Immune	4096	1024	32	32
Ferret 02	Normal Type C	32	128	32	32
	Immune	32	128	2048	1024
Patient B. P.	Acute	16	64	128	128
	Conv.	128	512	1024	1024

*Antibody titers are expressed as the reciprocal of the final serum dilution.

Confirmation of the types of virus present in these second passage fluids was obtained by hemagglutination inhibition tests using known paired ferret antisera. The tests were carried out by methods previously described.[4] Allantoic fluids from the B. P. No. 4 line, passed intra-allantoically, and amniotic fluid from the B. P. pooled fluids line passed with type A′ immune serum, were used as antigens. The control antigens were allantoic fluid of the Rhodes strain of type A′ and amniotic fluid of the JJ strain of type C influenza virus. The results are recorded in Table III. It is evident that the No. 4 fluid line is identified as influenza type A′ and the pooled fluids line as influenza type C.

That the patient from whom the throat washing was obtained was actually infected with both types of influenza virus, and that the dual isolation obtained was not the result of laboratory error, is also demonstrated in Table III. Significant rises in antibody titer to both type A′ and type C influenza virus are shown when the level of antibodies in the acute and convalescent serum of this patient are compared.

SUMMARY AND CONCLUSIONS

Isolation of both type A′ and type C influenza viruses from the same throat washing has been described. In addition it has been shown that the patient was infected simultaneously with both viruses.

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SIMPLIFIED TITRIMETRIC TECHNIQUES FOR THE ASSAY OF CALCIUM AND MAGNESIUM IN PLASMA

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THE quantitative determination of plasma concentrations of calcium and magnesium is of major importance in evaluating the anticoagulant action on human blood of ion-exchange resins.[1-3] The authors have found assay of the low concentrations of calcium or magnesium present after exposure of the plasma to ion exchangers unsatisfactory when dependent upon quantitative precipitation of the calcium. Such precipitation is an essential step in available chemical methods for the analysis of either cation.[4-11]

Schwarzenbach and his collaborators[12-17] have investigated many dyes which form soluble colored complexes with several metals. Aqueous solutions of these dyes at a critical pH have a characteristic color which changes in the presence of minute concentrations of the metals. Addition of sufficient ethylene diamine tetra acetate (EDTA) results in a reversion to the original color if the pH of the system is not altered.

Using these principles, methods for the analysis of plasma calcium and magnesium concentrations have been developed.[18] The chemical and technical details are described in the following report.

MATERIALS AND REAGENTS

All glassware is Pyrex and rendered metal-free by soaking in two normal nitric acid and rinsing with metal-free water until effluent rinse is neutral to methyl-red.[19]

All pH measurements are performed with the Beckman pH meter Model G (glass electrode), with use of the special glass electrode (type E) for pH values greater than 10.5. The electrodes and cup are washed with alkaline (pH 9 to 11) EDTA followed by metal-free water to insure freedom from contamination.

SOLUTIONS

Calcium Standard (100 millimolar calcium chloride).—Dissolve 1.001 Gm. of calcium carbonate in dilute hydrochloric acid and make up to 100 ml. with water. Subdilutions of this material are made to give desired calcium concentrations, and are stable below pH 5.0.

Magnesium Standard (100 millimolar magnesium chloride).—Dissolve 1.216 Gm. of metallic magnesium in concentrated hydrochloric acid (in a fume hood) and dilute to 500 ml. with distilled water. Subdilutions are made to give magnesium solutions of concentrations to be investigated, which are stable at pH below 5.0.

EDTA Titrating Solutions.—Dissolve 37.2 Gm. of di-sodium ethylene diamine tetra acetic acid-di-hydrate (EDTA) in approximately 600 ml. of water in a one liter volumetric

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This work was assisted by a grant-in-aid from the National Institutes of Health and by the National Blood Program of the American Red Cross.
Received for publication, April 20, 1951.

flask. The solution is titrated with 1.0 N NaOH to a pH of 10.5 to 11.0, and then made to one liter with distilled water. The titration requires approximately 200 ml. of the alkali. Subdilutions of this material are made as required with maintenance of the pH from 10.5 to 11.0 by addition of 1.0 N NaOH. These solutions are stable.

Ammonium Hydroxide-Ammonium Chloride Buffer.—One molar ammonium hydroxide is titrated with 1M ammonium chloride to pH 10.85. The pH of the volatile buffer is checked and adjusted at least weekly.

Potassium Cyanide.—Dissolve 45.56 Gm. KCN in one liter of water to make a 0.7 molar solution which has a pH of approximately 11.4. (Extreme caution must be used to avoid imbibition or inhalation of fumes from this solution.)

INDICATORS

Murexide (Calcium indicator).—Since this material is unstable in solution,[20] it is used in solid form. Approximately 0.20 Gm. murexide (ammonium purpurate) (obtainable from Eastman Kodak Co.) is mixed with 100 Gm. chemically pure fine-crystal sodium chloride.

Eriochromschwarz T (Magnesium indicator).—Ten grams of 4 sulfo-5 nitro-2 hydroxy naphthalene azo naphthol (C.I. 203) (obtainable from W. A. Taylor & Co.) are dissolved in approximately 250 ml. metal-free water; 10 ml. one normal sodium carbonate are added, and the solution brought to one liter with isopropyl alcohol. The solution is filtered through Whatmann No. 1 paper in a buchner funnel, and the filtrate is used as the indicator.[20, 21] This solution is stable for many weeks at 4° C.

PROCEDURE

Pipettes calibrated to deliver are used in order to avoid exhaled carbon dioxide.

It has been found that the solutions containing the metal and dye system to be titrated as well as metal-dye solutions containing an excess of EDTA are useful guides to the approaching end point during titration of unknowns. Such solutions are freshly made and the dyes added as necessary.

In titrations involving relatively high concentrations of calcium or magnesium (0.5 millimolar) or, if protein is present, one must approach the end point very slowly, since a period up to forty-five seconds between drops of EDTA is required for the color to become stabilized.

When the end point of the titration is reached, a slight excess of EDTA is added to assure that no further color change will occur; the volume added prior to this excess is used in calculations.

Murexide Titration (calcium).—To the titration flask containing 3 ml. of the sample to be analyzed are added 1 ml. of 1.0 normal sodium hydroxide and 1 ml. of 0.7M potassium cyanide. Approximately 0.04 Gm. of the murexide-sodium-chloride mixture is added with a spatula and the solution is titrated with an appropriate concentration of EDTA. The metal-free murexide solution at the pH of the titration is lilac-lavender, and in the presence of unchelated calcium ions it assumes a primrose-pink shade. This is true both in protein-free solutions and in plasma. The end point therefore occurs when there is no further color change toward the lavender, and is clearest if the EDTA concentration is such that one to two milliliters are required.

Eriochromschwarz Titration (calcium plus magnesium).—To a 3 ml. sample of plasma or serum are added approximately 0.12 ml. (calibrated dropper) of 1.0 normal sodium hydroxide, 1.0 ml. of ammonium-hydroxide-ammonium-chloride buffer, 1.0 ml. of potassium cyanide solution, and approximately 0.14 ml. (two drops) of Eriochromschwarz indicator, and the sample is titrated. For samples containing no protein, the sodium hydroxide is omitted. At this pH, metal-free Eriochromschwarz is blue in protein-free solutions and emerald green in protein solutions. In the presence of unchelated magnesium it assumes a royal red color. The end point in protein-free solution is reached when there is no further color change toward the blue, whereas the end point in plasma titrations is no further color change toward the green.

It has been found possible to perform both titrations on one sample by first doing the murexide titration and then lowering the pH to less than 3.0 which causes destruction of the murexide color[17] without destroying the activity of EDTA. The solution is then buffered for the Eriochromschwarz titration, and the addition of EDTA continued until the end point is reached. This modification is impractical in the presence of proteins, but it has proved useful in work with tissue ashes.

Calculations.—One mole of ethylene diamine tetra acetate is said to chelate one mole of divalent cation.[22-24] Experimentally determined ratios of EDTA to cation are, however, used in the experiments reported below. The following formulas have been employed in the calculation of results.

$$K = \frac{D \times E}{A \times C} \tag{1}$$

$$R = \frac{F \times G}{(A + B)C} \tag{2}$$

$$A = \frac{D \times E}{K \times C} \tag{3}$$

$$A + B = \frac{F \times G}{R \times C} \tag{4}$$

$$B = \frac{F \times G}{R \times C} - \frac{D \times E}{K \times C} \tag{5}$$

Where: K is a ratio of total EDTA moles to total calcium moles when the end point is reached in the murexide titration.

R is the ratio of total EDTA moles to total calcium plus magnesium moles when the end point is reached in the Eriochromschwarz titration.

A is the molecular concentration of calcium in the unknown.

B is the molecular concentration of magnesium in the unknown.

C is the volume of the unknown.

D is the molecular concentration of EDTA used in the murexide titration.

E is the volume of EDTA used to reach the end point in the murexide titration.

F is the molecular concentration of EDTA used in the Eriochromschwarz titration.

G is the volume of EDTA used in the Eriochromschwarz titration.

K and R are determined experimentally for various values of A and/or B.

Formulas 3, 4, and 5 are algebraically derived from formulas 1 and 2.

TABLE I. DETERMINATION OF MUREXIDE TITRATION RATIO (K)

	RANGE OF VALUES INVESTIGATED				OBSERVED VALUES			
	CONCENTRATION OF Ca	VOLUME	TOTAL Ca	CONCENTRATION OF Ca AFTER TITRATION	NUMBER	RANGE	MEAN	S.D. OF K
	A	C	A × C	A × C/C′	n	k	K	σ
SERIES	MILLIMOLAR	ML.	MOLES × 10^{-6}	MILLIMOLAR				
2	0.1009	3.0	0.3027	0.0390 to 0.0445	7	0.765 to 0.907	0.832	±0.051
2	0.1009	3.0	0.3027	0.0480 to 0.0490	3	0.815 to 0.915	0.864	±0.042
3	0.2018	3.0	0.6054	0.0810 to 0.0840	6	0.910 to 0.993	0.961	±0.037
4, 5, 6, 7, 8	0.5045 to 10.09	3.0	1.5135 to 30.27	0.1970 to 4.030	19	0.960 to 1.087	0.991	±0.030

TABLE II. DETERMINATION OF ERIOCHROMSCHWARZ TITRATION RATIO (R)

	RANGE OF VALUES INVESTIGATED							OBSERVED VALUES			
	CONCENTRATION OF Ca	CONCENTRATION OF Mg	SUM OF Ca AND Mg CONCENTRATION	VOLUME	TOTAL Ca	TOTAL Mg	CONCENTRATION OF Ca AND Mg AFTER TITRATION	NUMBER	RANGE	MEAN	S.D. OF R
	A	B	A + B	C	A × C	B × C	(A + B)C/C′	n	r	R	σ
SERIES	MILLIMOLAR			ML.	MOLES × 10^{-6}		MILLIMOLAR				
9	0	0.05005	0.05005	3.0	0	0.15015	0.0217 to 0.0224	9	0.730 to 0.787	0.759	±0.023
10 14a	0 to 0.0378	0.0501 to 0.1001	0.0879 to 0.1001	3.0 to 4.0	0 to 0.1512	0.2004 to 0.3003	0.0403 to 0.0422	12	0.877 to 0.979	0.923	±0.027
14a 15 16	0.0404 to 0.0757	0.0501 to 0.06006	0.1005 to 0.1258	3.0 to 4.0	0.1212 to 0.3028	0.1802 to 0.2004	0.0455 to 0.0622	12	1.025 to 1.092	1.057	±0.023
11, 11a, 12, 12a, 13, 13a, 14a, 14b, 15	0 to 7.568	0.0334 to 10.01	0.2002 to 10.01	3.0 to 4.0	0 to 30.27	0.1001 to 40.04	0.0780 to 3.950	71	0.960 to 1.080	1.031	±0.024

In calculation of the concentrations of calcium and/or magnesium present at the end of the titrations, the following notations have been used:

C′ is the total volume after titration (C plus volumes of buffer, cyanide, and EDTA).

$\frac{A \times C}{C'}$ is the concentration of calcium in the titrated solution at the end point.

$\frac{(A + B)C}{C'}$ is the sum of the concentrations of calcium and magnesium in the titrated solution at the end point.

EXPERIMENTAL

Determination of K and R.—Aqueous solutions with known calcium and calcium plus magnesium concentrations were analyzed by the murexide and Eriochromschwarz titrations, respectively. The results, shown in Tables I and II, were used to compute K and R.

Effect of Interfering Ions.—A series of analyses similar to those for the determination of K and R were performed in the presence of iron, copper and zinc concentrations in excess of those which would be expected in normal human plasma prepared for titration as described. In the case of the muerxide titration magnesium was also introduced. Results are in Table III.

TABLE III. EFFECT OF INTERFERING CATIONS ON TITRATIONS IN AQUEOUS SOLUTION

DETERMINATION	CONCENTRATION INTERFERING CATIONS	SERIES	VOLUME	PREDICTED VALUES	NO. OF OBSERVATIONS	OBSERVED DEVIATIONS FROM PREDICTED VALUES		
			C	RANGE	n	RANGE	MEAN	S.D. OF MEAN
	MILLIMOLAR		ML.	MILLIMOLAR		% OF PREDICTED VALUE		σ
A	Fe = 0.033	32	6.5	0.0926	2	+16.0	+16.0	
	Zn = 0.024	33	6.5	0.233	2	+10.3	+10.3	
	Cu = 0.031 Mg = 0.771	34 35	6.5	0.463 to 0.926	4	+1.51 to +4.55	+3.46	
A + B	Fe = 0.036	29	6.0	0.05005	5	+2.70 to +5.90	+4.42	±1.43
	Zn = 0.026 Cu = 0.034	20, 21 22, 23	6.0	0.8382 to 2.347	14	−4.56 to +3.95	−1.26	±2.71

A series of analyses were performed in the presence and absence of cyanide ion in order to evaluate its effect on the titration.

Recovery of Calcium and Magnesium from Plasma.—Plasma from whole blood previously exposed to Na-cycle sulfonated polystyrene cation exchanger (Dowex-50) was used. Spectroscopic examination of such plasma[25] reveals more than 95 per cent of the calcium and magnesium to be removed without significant reduction of the iron, copper, or manganese concentrations, and radio tracer techniques[3] show the zinc concentration to be unaltered.

The plasma was then exposed to fresh cation exchanger which had been regenerated with 1.8 molar NaCl and washed with 0.15 molar NaCl. On the addition of either indicator to properly buffered aliquots of this plasma the solution acquired the color of the metal-free dye.

To 100 ml. of plasma so prepared were added varying amounts of calcium and magnesium in the presence of heparin and the resultant solutions were analyzed. Results are shown in Table IV.

TABLE IV. RECOVERY OF KNOWN AMOUNTS OF CA AND MG FROM RESIN-TREATED PLASMA

	TOTAL ADDED TO PLASMA		DETERMINATION	PREDICTED VALUE	OBSERVED VALUES			
	Ca	Mg			NUMBER	RANGE	MEAN	S.D. OF MEAN (σ)
SERIES	MOLES × 10^{-6}			MILLIMOLAR	n	MILLIMOLAR		
40	12.108	6.006	A	0.1211	10	0.1175 to 0.1540	0.1335	±0.0128
			A + B	0.1811	6	0.1745 to 0.1915	0.1829	±0.0059
41	50.45	30.03	A	0.5045	10	0.4830 to 0.5580	0.5220	±0.0222
			A + B	0.8048	10	0.7920 to 0.8610	0.8210	±0.0220
42	302.7	100.1	A	3.027	10	2.902 to 3.180	3.042	±0.1064
			A + B	4.028	10	3.940 to 4.235	4.092	±0.0952

RESULTS

Determination of K (Murexide Titration) and R (Eriochromschwarz Titration).—In Tables I and II are given data from which values for K and R (formulas 1 and 2 above) have been computed. K and R remain constant over a large range, but below critical concentrations of the two metals, the values are seen to be dependent on the calcium and the sum of the magnesium and calcium concentrations, respectively. Examination of the data shows no independent relation of R to the concentration of either one of the metals.

On semilogarithmic coordinates, in Figs. 1 and 2, the concentration of the metals computed to be present at the end point of the titration $\left[\frac{A \times C}{C'}\right.$ and $\left.\frac{(A + B)C}{C'}\right]$ are recorded on the ordinate. The mean values for K and R are plotted as solid vertical lines over the range of concentrations in which they are constant, and as points for lower concentrations. The broken lines represent two standard deviations from the mean ($\pm 2\sigma$). Values of zero for K and R are the concentrations at which no color complexes with the dyes were visibly detectable.

We have found reliable titrations of nonprotein solutions with initial concentrations of calcium greater than 0.1 millimolar, and total concentrations of Ca plus Mg greater than 0.05 millimolar, and magnesium greater than 0.04 millimolar.

Use of Experimental Ratios in Calculating Calcium and Magnesium Concentrations.—In the subsequent results ratios have been used in the computations. The approximate concentration of calcium or magnesium in the unknown is obtained on inspection by substituting a value of 1.0 for K and R in formulas 3 and 4. From these approximations and the conditions of the titration, values for $\frac{A \times C}{C'}$ and $\frac{(A + B)C}{C'}$ are estimated, and the proper ratios

obtained from Figs. 1 and 2. Substitution of correct values for K and R in formulas 3 and 4 leads to more precise estimations.

Effect of Interfering Ions on Calcium and/or Magnesium Analyses.—In Table III it is shown that iron, zinc, copper, and magnesium at the concentrations investigated do not significantly alter the results except in the murexide titration when the calcium concentration is less than 0.5 millimolar, which is presumably the result of the very high magnesium-calcium ratio.[12, 15, 16] The concentrations of iron, zinc, and copper investigated do not apparently alter the Eriochromschwarz titration.

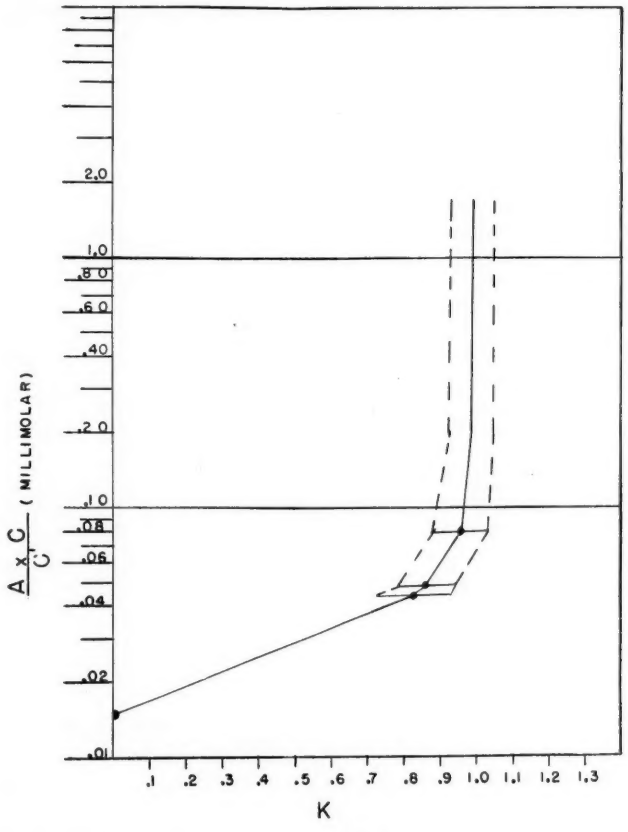

Fig. 1.

If the cyanide is not added to the system, marked interference is found in both titrations with these concentrations of copper, zinc, or iron, but no variations in either the murexide or Eriochromschwarz titration could be attributed to the presence or absence of the cyanide ion alone.

Citrate, phosphate, carbonate, and borate buffers have been found to interfere with both titrations, but the concentration of these anions found in normal plasma does not appear to cause such interference.

The dry-ashing of biologic tissue has led to marked anionic interference in the titrations which is probably the result of conversion of organic phos-

phorus to inorganic phosphates. Removal of interfering anions from tissue ashes by means of anion exchangers has been found possible, but has not increased the sensitivity or accuracy of plasma analyses.

Effect of pH.—The optimal pH values for the titrations were found to be greater than 12.4 for the murexide and between 10.8 and 11.2 for the Eriochromschwarz.

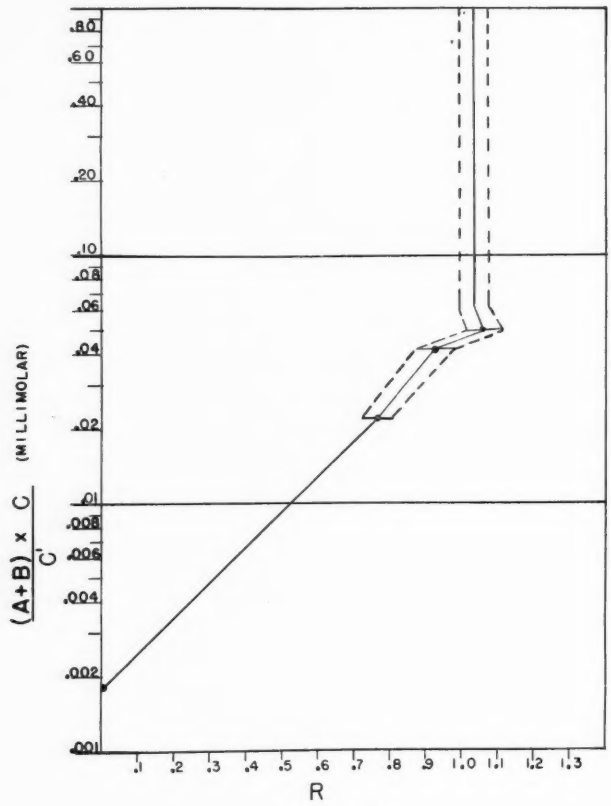

Fig. 2.

Assay of Calcium and/or Magnesium in Plasma.—The murexide titration for plasma samples containing more than 1.0 millimolar calcium is enhanced by dilution of the plasma with an equal volume of 0.10 molar sodium chloride and titrating the diluted plasma as above.

In Table IV are shown the plasma recovery data. The assay value is compared with the expected values. It should be noted that at none of the concentrations investigated did the mean of the determined values vary from the predicted values by more than one standard deviation of the mean of the observed values. From the range of values shown and their distribution as expressed by the standard deviation of the mean, evidence as to the sensitivity and reproducibility of the methods is available.

DISCUSSION

Murexide forms a colored complex with magnesium which is not so stable or intense as the calcium-murexide complex.[12, 15] Eriochromschwarz forms a more stable and intensely colored complex with magnesium,[13, 14] thus requiring the presence of magnesium (more than 0.02 millimolar) for its use as an indicator.

Since EDTA has approximately one hundred times greater affinity for calcium than for magnesium,[22] chelation of the calcium will occur before that of magnesium in the two titrations outlined. Thus, the color shift is observed in the murexide titration when sufficient EDTA is present to chelate all the calcium, and in the Eriochromschwarz titration when sufficient to chelate both the calcium and magnesium.

Although certain cations found in plasma have higher affinity for EDTA than either calcium or magnesium[22, 23, 26, 27] the data reported indicate that the cyanide and/or plasma proteins prevent interference from those present in relatively high concentration (Fe, Zn, Cu).

The main advantages of the murexide titration over oxalate methods are its rapidity and technical simplicity, wide range of calcium concentration over which effective, and reliability even in presence of proteins concerned with coagulation. The disadvantages of the technique stem primarily from the instability of the dye and an end point which is not extremely sharp. No comparison with flame photometric techniques has been made.

The Eriochromschwarz titration is seen to be more accurate than the murexide. In many instances the sum of the calcium and magnesium concentrations may suffice.[28] Since no simple, reliable method exists for the determination of magnesium in plasma, the combination of the Eriochromschwarz titration with any analytic method for calcium would provide an estimate of the magnesium. Such a method is subject to the limitations of any indirect method, but, as shown in the above data, the assay of the sum of calcium and magnesium is quite accurate over a large range.

The instability of the murexide and complexity of the Eriochromschwarz color combinations have apparently precluded direct colorimetric techniques.

The use of experimentally determined titration ratios corrects for any error in the titrating solution. The difference between the observed values for K and R is probably significant, since the same titrating solutions and standard metal solutions were used throughout. A similar difference has been reported,[29] but more specific chelating agents[30] have not been investigated.

SUMMARY

Titrimetric techniques with colorimetric end points for the direct assay in plasma of calcium and the sum of calcium and magnesium concentrations are described.

The techniques have been found reliable with concentrations in plasma of the two cations ranging from 0.10 millimolar for calcium and 0.18 millimolar for the sum of calcium and magnesium to the clinically significant levels of 3.0 millimolar and 4.0 millimolar.

The reproducibility of the calcium titration may be somewhat less than that reported for the oxalate methods when normal serum calcium concentrations are involved. The range and sensitivity of the technique, however, are greatly in excess of that reported for oxalate methods.

The titration for the sum of calcium and magnesium is simple, accurate, and extremely sensitive in comparison to previously reported methods for the direct assay of plasma magnesium thus providing, by its combination with a calcium analysis of any type, an indirect method for the estimation of plasma magnesium.

Such techniques permit the study of calcium and magnesium concentrations in normal or pathologic plasma, and further enable investigation of kinetics and equilibria involved in the use of cation exchangers as whole blood anticoagulants.

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THE SPECIFICITY OF THE DESICCATION METHOD FOR DETERMINING ALCOHOL IN BIOLOGICAL FLUIDS

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THE concentration of alcohol in biological material must be determined in some special research problems and in some routine medicolegal investigations; in the former, emphasis is placed on the sensitivity and precision of the method, while in the latter the simplicity, reliability, and specificity for alcohol are paramount; a method which would serve either purpose adequately would be most desirable. A review of the available methods described in the literature and experimentation with nine of these (Blotner,[4] Friedmann and Klaas,[5] Friedmann,[6] Gaudry,[7] Gettler,[8] Hambersin,[11] Harger,[12] Widmark,[22] and Winnick,[23] established the conviction that the desiccation procedure developed by Widmark offered the greatest possibility of devising a method having this dual capacity.

In the Widmark method, the sample is dessicated in one compartment of a closed flask; in the other compartment the alcohol from the sample is oxidized using dichromate in sulfuric acid. When this operation has been completed, the oxidizing reagent may be titrated in the same flask. Using a series of these flasks, many determinations of alcohol can be made at one time in a minimum of laboratory space. However, using the Widmark method, which employs concentrated sulfuric acid in the oxidizing mixture, workers have reported many high values attributable to nonalcoholic interfering materials (Hinsberg,[14, 15] Heilner,[13] Widmark, cited by Gisselson[9]). The method to be reported is based on studies of specificity for alcohol which demonstrated that spuriously high values could be avoided by using diluted acid in the oxidizing reagent. This modification, and one which ensures a "zero" blank at all times, have improved the specificity for alcohol and the precision of the desiccation method without affecting its simplicity.

MATERIALS AND METHODS

Apparatus.—Flasks similar to the one shown in Fig. 1 are used for the analysis. These are standard taper, wide-mouth, 50 ml. Erlenmeyer flasks which may be calibrated to contain 50 milliliters. A glass cup (1.2 ml. capacity) and stem are sealed to the solid glass stopper, which may be held in place with a rubber band, as shown in the figure. An all-glass automatic micro burette equipped with reservoir is necessary for storage and delivery of the oxidizing solution (Neugebauer[20]). This burette should be fitted with a capillary tip to control the rate of delivery of the oxidizing solution (1 ml. in twenty seconds).

Reagents.—Distilled water: Distilled water is redistilled after the addition of 1 Gm. NaOH and 0.5 Gm. $KmNO_4$ per liter.

Oxidizing solution: 400 ml. 24 N-H_2SO_4 is added to 200 ml. redistilled water containing 0.9 Gm. $K_2Cr_2O_7$. This solution is heated for one hour at 80° C. and then cooled (Martin

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A portion of a thesis submitted as partial requirement for the degree of Doctor of Philosophy in the University of Toronto.
Received for publication, June 25, 1951.

Du Pan[18]). After adjusting the acid concentration to 15 N, this solution is stored in the automatic burette.

Ferrous Ammonium Sulfate: 2.5 Gm. $FeSO_4(NH_4)_2SO_4.6H_2O$ and 50 ml. concentrated H_2SO_4 per liter of distilled water. The exact alcohol equivalent of this solution is determined by analyses of known concentrations of alcohol.

Orthophenanthroline Ferrous Complex B.D.H.: 1 ml. diluted to 50 ml. with distilled water.

Diphenyl Carbazide B.D.H.: 0.5 ml. diluted to 50 ml. with 95 per cent ethanol freshly prepared (Rowland[21]).

Precautions.—All glassware must be cleaned immediately before use in concentrated chromate cleaning solution (caked blood may be removed by rinsing in 20 per cent KOH and then in water). Glassware is then thoroughly rinsed with distilled water and finally with redistilled water. Pipettes may be suction dried.

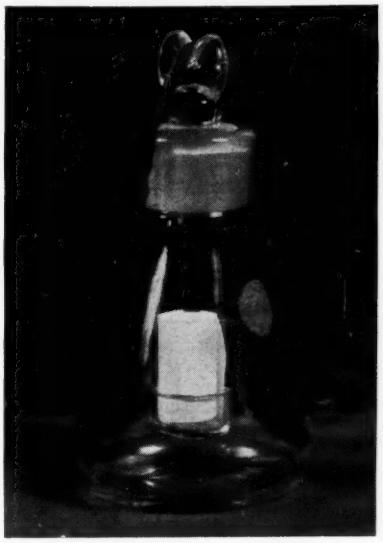

Fig. 1.—Desiccation flask (with filter paper in place).

Procedure.—Three milliliters of oxidizing solution is added to each flask. A half sheet of 7 cm. filter paper (No. 42 Whatman trimmed to 2 cm. width) is rolled around the stem of the cup and 0.5 ml. of the solution to be analyzed (redistilled water is used as a blank) is pipetted into the cup. The filter paper is then slipped down into the cup (it is held in place by the side of the cup and provides a large evaporating surface for the sample [Abels[1]]). The stopper and cup are quickly placed in the flask and held in place with a rubber band. The flasks and contents are heated in an oven at 60° C. (±2° C.) for ninety minutes (± five minutes) for a 0.5 ml. sample or for one hundred and fifty minutes for a 1.0 ml. sample. Alternatively, the flasks may be left overnight (twelve to sixteen hours) at room temperature (20 to 24° C.). The flasks are then chilled for ten minutes in a refrigerator, the stopper and sample cup are removed, and 20 ml. redistilled water added. The dichromate remaining in the flask can either be determined by titration or colorimetrically.

Titration.—Standardized ferrous ammonium sulfate is added to the contents of the flask until faint blue color appears; 5 drops of orthophenanthroline are added, and the titration continued until a pink color becomes permanent. The alcohol concentration equals the number of milliliters to titrate the blank minus the number of milliliters to titrate the sample multiplied by the alcohol equivalent per milliliter of the ferrous ammonium sulfate solution used.

Colorimetric determination.—The contents of the flasks are diluted to 50 ml. with distilled water; 2.0 ml. is pipetted (pipettes rinsed several times with the solution to be measured) into a 100 ml. volumetric flask containing 0.4 ml. 24 N H_2SO_4 and approximately 50 ml. distilled water. Then 1 ml. diphenylcarbazide (Rowland[21]) solution is added and the contents are diluted to 100 ml. and mixed. Approximately 10 ml. of this solution are placed in matched colorimeter tubes and read in the Evelyn Colorimeter using filter 540. The galvanometer is set to 100, using a solution of 0.4 ml. 24 N H_2SO_4, 1 ml. diphenylcarbazide in 100 ml. of water. The concentration of alcohol in the sample may be obtained from the following equation:

Concentration of alcohol (parts per 100 for 0.5 ml. sample) = 0.234 ("L" blank–"L" sample) where "L" = 2– log galvanometer reading.

RESULTS

Table I illustrates the recovery of alcohol added to blood or urine using this desiccation method.

Specificity.—The conditions of temperature and acid concentration chosen for this method are such that acetone does not interfere, yet alcohol is quantitatively oxidized. The use of the glass sample cup precludes the possibility of contamination of the oxidizing solution by bits of filter paper or dried sample. In order to indicate the specificity of this method for alcohol, samples were obtained from diabetic dogs and persons. These samples included blood and urine samples from four depancreatized dogs deprived of insulin for as long as twelve days (Co_2 combining power 26 volumes per cent) and from four patients in diabetic acidosis (CO_2 combining power 30 volumes per cent) as well as samples of urine from thirty uncontrolled diabetics in the hospital. These studies indicated that

TABLE I. THE PRECISION OF THE DESICCATION METHOD FOR DETERMINING ALCOHOL IN BIOLOGICAL FLUIDS

SAMPLES	NO. OF SAMPLES	ALCOHOL ADDED (PARTS PER HUNDRED)	ALCOHOL RECOVERED ±S.D.* (PER CENT)	REMARKS
Urine 0.5 ml.	10	0.081	100.4 ± 1.3	Heating to 60° C. colorimetric
	10	0.161	101.3 ± 0.5	Heating to 60° C. colorimetric
	10	0.322	101.1 ± 1.8	Heating to 60° C. colorimetric
Blood 0.5 ml.	4	0.157	98.7 ± 0.3	Heating to 60° C. Titration.
	5	0.205	98.3 ± 0.3	These low recoveries can be corrected by standardizing titrating fluid against known concentrations of alcohol.
	5	0.310	96.3 ± 2.2	
Blood 0.5 ml.	4	0.157	100.4 ± 3.1	
	5	0.310	100.2 ± 0.4	Heating to 60° C. colorimetric. Applied to protein-free supernatant obtained after addition of $HsSO_4$ and KOH to blood or urine.
Blood 1.0 ml.	5	0.157	100.0 ± 1.5	
	5	0.157	100.3 ± 1.2	
	5	0.204	100.1 ± 0.6	
Urine 0.5 ml.	5	0.418	99.2 ± 0.9	
Urine 1.0 ml.	5	0.084	98.3 ± 1.6	
Blood 0.5 ml.	10	0.153	99.4 ± 1.5	Heated to 60° C. colorimetric.
	10	0.153	98.9 ± 1.8	Left overnight. First trial of method by a technician.
Total	98	Average	99.6 ± 1.3	(per cent of the concentration added)*

*For a blood or urine concentration of 0.150 per cent, this S.D. (1.3 per cent of the concentration added) is equivalent to 0.002 parts per hundred.

using this method, the maximum values of "apparent" alcohol in diabetic acidosis are at most 10 mg. per 100 ml. in blood, and at most 15 mg. per 100 ml. in urine. These values probably represent actual concentrations of alcohol. Studies indicated that alcohol may be formed in samples which contain high concentrations of glucose as a result of fermentative action, when these samples are inefficiently preserved. The preservative suggested by Marron[17] proved to be very efficient (75 mg. sodium fluoride and 50 mg. sodium citrate for each 10 ml. of blood or urine). These, and other studies indicated that there are three possible explanations for the high urine or blood values of "apparent" alcohol reported in some cases of diabetic acidosis (Friedmann,[5] Widmark, cited by Gisselson,[9] Blotner,[4] and Bagchi[3]). These high "apparent" alcohol values could be explained (a) by the harsh conditions chosen for the oxidation of alcohol, (b) by surreptitious ingestion of alcohol by the patients, or (c) by inefficient preservation of the samples.

Using this desiccation method, the "apparent" alcohol values in blood due to paraldehyde in therapeutic doses (8 ml.) was of the order of 5 mg. per 100 ml.; similarly the "apparent" alcohol values in urine due to urotropin given to patients in a dose of 4 Gm. four times a day, was of the order of 16 mg. per 100 ml. These values were almost doubled when the proteins and ketones were precipitated in these samples, using the reagents Friedmann[6] employed with saliva. In these experiments the concentration of alcohol was determined using a sample of the clear protein-free supernatant liquid obtained by addition of these reagents to blood or urine and centrifuging. This experience indicated that it is preferable to gain the necessary specificity by adjusting the conditions for oxidizing alcohol rather than through the use of special reagents.

While ether in anesthetic concentrations interferes in this method, it is a highly unlikely source of error in samples taken from drivers of motor vehicles. As with other oxidation methods, alcohols other than ethyl may interfere. Where methyl alcohol is suspected, as in samples from embalmed tissues, other methods (e.g., Albernethy[2]) must be used. Within the limits indicated by these investigations blood and urine concentrations of alcohol can be accurately measured using this method, provided that the samples are properly collected and preserved.

DISCUSSION

None of the conditions for the oxidation of alcohol employed in this method are critical. The simplicity and adaptability of the procedure recommend it for routine use. The precision of this method appears to depend on (1) scrupulous cleanliness, and (2) the technique of preheating the oxidizing reagent before use. This technique (Koller,[16] and Martin Du Pan[18]) removes the traces of oxidizable materials always present, even in the best sulfuric acid obtainable. When prepared in this way, and stored in diffuse light, the oxidizing mixture gives the same negligible blank value over a period of several months. Since there is some reduction of dichromate in sulfuric acid, even in diffuse light (Meier,[19] Hallen[10]) the solution remaining in the burette portion of the storage vessel should be discarded each day before a series of analyses.

SUMMARY AND CONCLUSIONS

A desiccation method for determining alcohol has been described, and its specificity for alcohol investigated. While the precision of this method makes it suitable for research purposes, its simplicity, reliability, and specificity for alcohol make it suitable for routine medicolegal work.

These studies indicate several possible explanations for the high values of "apparent" alcohol found by some workers in blood and urine of persons with diabetic acidosis.

My thanks are due to Professors J. K. W. Ferguson and G. H. W. Lucas for their many constructive suggestions during this research and in preparing this paper.

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A CALIBRATOR FOR DETERMINING THE VOLUME OF THE WARBURG VESSEL PLUS MANOMETER

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IN CALCULATING the constant for a Warburg Manometer it is necessary to know the volume of the vessel plus the volume of the manometer to the 150 mm. mark. A previously described micrometer calibrator[1] provided a simple and direct method for the determination of the volume of the manometer. The volume of the vessel, however, had to be determined separately by conventional methods. Scholander and associates[2] calculated the volume of the manometer plus vessel by adding a known amount of gas to the system, and determined the resulting pressure change as measured on the Warburg Manometer. The volume of the vessel plus manometer was then calculated by an equation using the barometric pressure and the vapor pressure of water.

In the method herein described the volume of the manometer plus vessel can be read directly from the calibrator. When sufficient gas is removed from the manometer plus vessel to reduce the pressure within the manometer to a predetermined value, the volume of the vessel plus manometer equals 100 times the volume of gas removed. The desired pressure reduction is calculated from an equation using the barometric pressure and the vapor pressure of water. Once this value is determined, it suffices for a series of calibrations.

METHOD

Theoretical Considerations: Boyle's law states that

$$PV = \text{Constant} \qquad \text{(Equation 1)}$$

where P is the pressure of the gas and V is its volume. If the volume of a given amount of gas is increased by v, the pressure will be decreased by a corresponding amount, p. If the gas is exposed to water vapor it is necessary to correct for the partial pressure of water. Thus the relation between the final pressure and volume and the initial pressure and volume is given by equation 2:

$$(P_B - P_w)\ V = (P_B - P_w - p)\ (V + v) \qquad \text{(Equation 2)}$$

where P_B is the barometric pressure, P_w the vapor pressure of water at the temperature T, V the initial volume of the gas, i.e., the volume of the manometer plus vessels, p the change in pressure, and v the change in volume. Solving equation 2 for the volume of manometer plus vessel we get:

$$V = \frac{v\ (P_B - P_w - p)}{p} \qquad \text{(Equation 3)}$$

Since we wish the volume of the manometer plus vessel to be exactly 100 times the volume of the gas removed we must choose an appropriate value of p so that:

$$\frac{(P_B - P_w - p)}{p} = 100 \qquad \text{(Equation 4)}$$

From the Department of Anatomy, Western Reserve University School of Medicine.
Aided by a grant from the National Foundation for Infantile Paralysis.
Received for publication, June 30, 1951.

solving equation 4 for p we get:

$$p = \frac{P_B - P_w}{101} \qquad \text{(Equation 5)}$$

Since we must read the pressure change in millimeters of Brodie's solution on the Warburg Manometer, and since one millimeter of mercury is equal to 13.17 millimeters of Brodie's solution, i.e. (the density of mercury [13.6] divided by the density of Brodie's solution [1.033]) equation 5 becomes:

$$p = .130(P_B - P_w) \qquad \text{(Equation 6*)}$$

P_B is the barometric pressure in millimeters of mercury. (A temperature correction for the observed barometer reading amounts to about 0.4 per cent at 25° C. and the appropriate value [see *Handbook of Chemistry and Physics*] should be subtracted from the observed barometer reading.) P_w is the vapor pressure of water in millimeters of mercury at the temperature used in the calibration and p is the desired pressure change in millimeters of Brodie's solution.

PRACTICAL CONSIDERATIONS

The Warburg vessel, *W* (Fig. 1), is attached to the manometer. The vent, *V*, of the side arm is opened, and the vessel is placed in a water bath at room temperature (or at any desired fixed temperature). A short period is allowed for temperature equilibration, since handling the vessel may change its temperature. The previously described calibrator,† *A*, is filled with water and a glass adapter, *B*, with a male standard taper joint, is inserted into the calibrator and held in place by means of the spring clips, *D*. The calibrator is placed in a vertical position, adapter end up (not illustrated) and all of the air is removed from the calibrator chamber by advancing the plunger, *P*. The glass adapter is inserted into one of the "V" grooves of the clamp, *E* (Figs. 1 and 2), and fixed in place by means of the wing nut, *F*. A small segment of amber gum rubber tubing, *G*, is placed over the end of the adapter. The vertical segment, *H*, above the manometer stopcock is moistened with water and attached to the adapter as shown in the figures by inserting it into the rubber tube, *G*. The calibrator is clamped to the manometer by tightening the wing nut, *J*. To minimize trapping of air in the rubber segment, *G*, it is preferable to have ends of the manometer and the adapter practically touching.

The plunger, *P*, of the calibrator is advanced until the capillary tube of *H*, and the bore of the stopcock, *S*, are just filled with water. This is dead space and is not part of the volume used in the calculation. The Brodie's solution in the manometer is adjusted to the 150 mm. mark by turning the screw clamp at the bottom of the manometer and the manometer vent, *V*, is closed.‡ Both arms should now read 150 millimeters. If the level of liquid is not exactly equal on the two sides it may be adjusted by means of the calibrator. After the initial reading of the calibrator is recorded, sufficient gas is removed to make the difference in pressure between the right and the left limbs of the manometer equal to the value of p as calculated by equation 6. When the level of Brodie's solution in the right limb of the manometer is maintained at the 150 mm. mark and the level in the left limb reads 150 minus p, the final reading of the calibrator is recorded. With a little practice the adjustments of the calibrator and manometer can be made in less than one minute.

Since the diameter of the calibrator plunger has a cross-sectional area of 1.00 square centimeter, each millimeter advancement of the plunger (two complete revolutions) corresponds to a volume displacement of 0.100 ml. Since the volume of the vessel plus manometer is 100 times the gas volume removed, each millimeter advancement of the plunger corresponds to 10.0 milliliters.

EXAMPLE

When the barometric pressure was 749.2 mm. Hg, and the temperature was 25° C., the temperature correction factor for the barometer at 25° is −3.0 mm. Hg, and vapor pressure of

*In practice equation 7 is used (see discussion).

†Available through the Micro-Metric Instrument Co., Cleveland, Ohio.

‡If the vessel side arm does not have a vented stopper, venting is accomplished through the manometer stopcock side tube.

H_2O is 23.8 mm. Hg. The requisite pressure difference in millimeters of Brodie's solution necessary to make the calibration ratio equal to 100:1 is calculated from equation 6,* and it equals p = .130 (749.2 – 3.0 – 23.8) = 94.1 mm. of Brodie's solution. Thus, with the liquid in the right manometer limb set at 150, we wish the liquid in the left arm of the manometer to read (150 – 94.1) or 55.9 mm. The initial reading of the micrometer was 14.55 mm. The

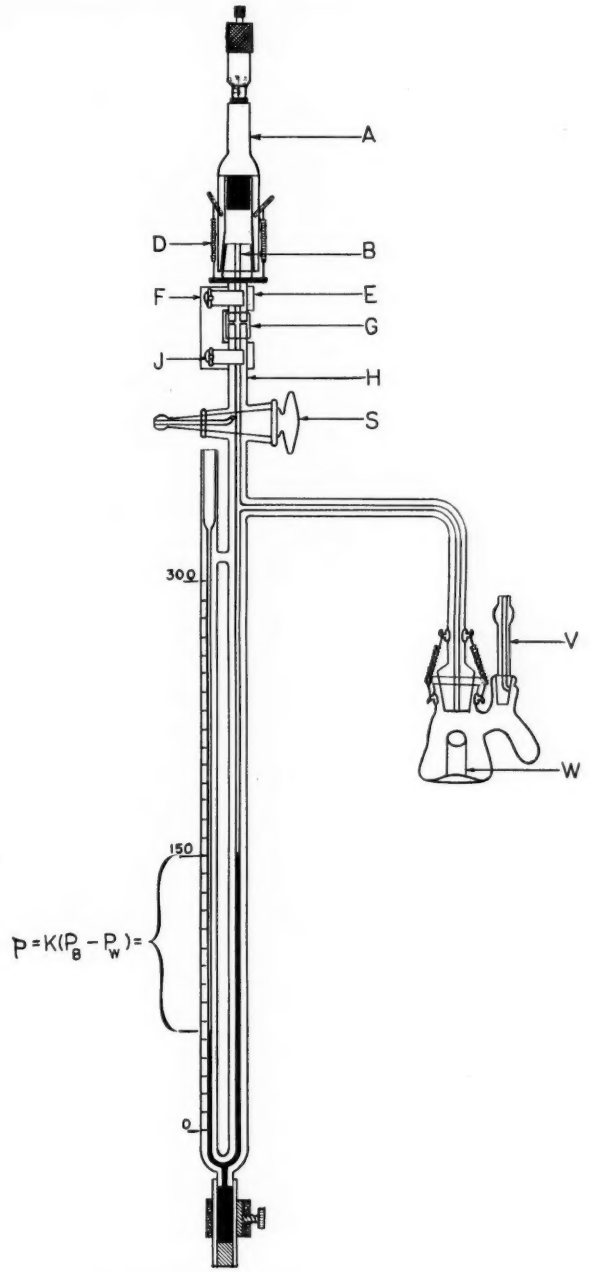

Fig. 1.—Calibrator attached to Warburg manometer.

*In practice equation 7 is used (see discussion).

final micrometer reading after the removal of sufficient gas to reduce the pressure by 94.1 mm. was 16.27. The amount of gas removed was 0.172 c.c., and the volume of the manometer plus vessel is equal to 100 times this value, or 17.2 milliliters.

RESULTS

The results of a series of calibrations on nine manometers are shown in Table I.

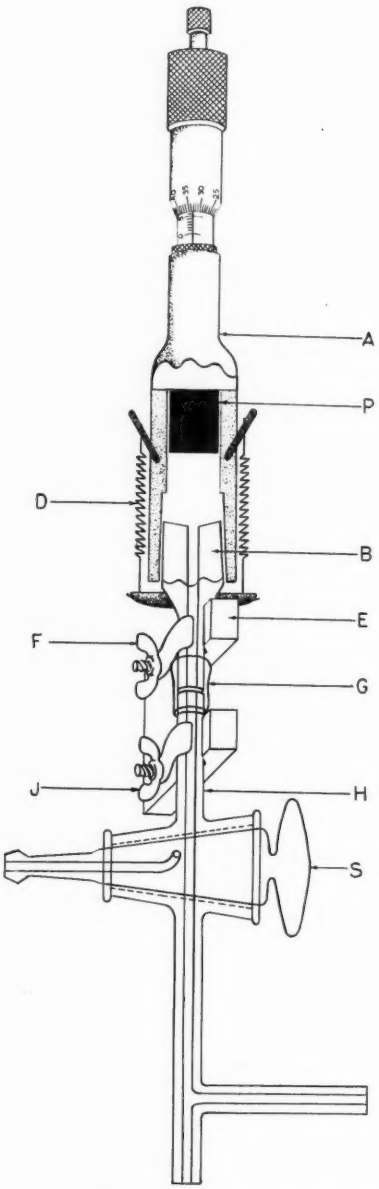

Fig. 2.—Enlarged view of calibrator attached to manometer.

TABLE I. DETERMINATION OF VOLUME OF WARBURG MANOMETER PLUS VESSEL
INDIRECT METHOD

Manometer no.	31	33	34	35	36	37	38	39	40
Vessel no.	51	52	53	A	56	57	58	59	60
Volume (c.c.)	19.2	16.8	15.0	20.1	17.2	18.0	15.9	14.6	20.1
	19.6	17.3	15.0	20.2	16.9	17.9	16.1	14.5	20.2
	19.3	17.2	15.0	19.6	17.0	17.9	16.1	14.6	20.2
	19.4	17.3	14.9	19.8		17.9	15.7	14.7	20.1
							16.0		
Average volume (c.c.)	19.4	17.2	15.0	19.9	17.0	17.9	16.0	14.6	20.2
Average error of replicates (%)	0.6	0.8	0.2	1.0	0.6	0.2	0.8	0.3	0.3
Maximum error of replicates	1.0	2.3	0.7	1.5	1.2	0.6	1.9	0.7	0.5
True volume (c.c.)	19.3	16.9	14.8	20.0	16.6	17.8	15.8	14.4	20.0
Error (from true value) (%)	+0.5	+1.8	+1.3	−0.5	+2.2	+0.6	+1.3	+1.4	+1.0

Average error of nine determinations = +1.0%.
Standard deviation of the error = ±0.7%.

DISCUSSION

The average error of the four or five replicate determinations for each manometer was less than 1 per cent. The maximum error of any one determination was less than 2 per cent. The average volume of the nine manometers was 1 per cent high and the standard deviation was ±0.7 per cent. The error for eight of the nine vessels ranged between +0.5 and +2.2 per cent.

Assuming that the true volume of the manometer is known to an accuracy of 0.25 per cent, and that the standard deviation of the true volume is twice this amount, the observed error of +1 per cent has real significance (p value less than 0.001). This +1 per cent error must be a constant error operative in all of the determinations.

Reconsidering the derivation of the equations, one source of error became apparent. All calculations were based on the assumption that the temperature of the gas remains constant throughout the calibration. If, however, sufficient time is not allowed for temperature equilibration to take place, the temperature in the Warburg vessel will decrease by about 1 per cent when its volume is increased by 1 per cent, and the observed pressure change in the manometer would be about 1 per cent less than that given in equation 6. Since the actual calibration is completed in less than one minute, it is most likely that temperature equilibration has not taken place within the vessel. Consequently, if sufficient gas is removed to make the pressure difference equal to that given in equation 6, the volumes would be about 1 per cent high. Dividing the constant in eqlation 6 by 1.01, we obtain the corrected equation 7.

$$p = .129\ (P_B - P_w) \qquad \text{(Equation 7)}$$

If we apply this correction to the values given in Table I, the determined volumes vary between −1.5 per cent and +1.2 per cent of the true value with seven of the nine values being less than 1 per cent. It is more convenient to proceed rapidly with the calibration using the corrected equation 7 than it is to allow sufficient time for temperature equilibration to be reached within the vessel.

The precise density of Brodie's solution is also critical to the calibration of the manometer volume. If the density is increased due to evaporation, errors

may be introduced in the calibration. Reference to equation 3 indicates that a 1 per cent increase in the density of the Brodie's solution would change the volume by approximately 1 per cent. Therefore, if the Brodie's fluid in the manometer had evaporated to 75 per cent its original volume, the density as well as the volume of the manometer would be increased by 1.5 per cent.

CONCLUSION

A rapid method for the determination of the volume of the Warburg Manometer plus vessel is described. When sufficient gas is removed from the manometer to reduce the pressure by a predetermined amount (as calculated by a simple equation) the volume of the vessel plus manometer is equal to 100 times the volume of gas removed. The volumes can be read directly from the calibrator. The method is accurate to less than 1.5 per cent.

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MICRO-CONICAL TIPPED HOMOGENIZER AND ITS USE IN ANALYTICAL PROCEDURES

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POTTER and Elvehjem[1] introduced an all-glass tissue grinder for homogenizing tissue. Although it is possible to make an ultramicro homogenizer of similar design, certain drawbacks are encountered when volumes of 50 μl or less are employed because of fluid adsorption on the ground glass surfaces. The micro-conical tipped modification herein described minimizes this fluid loss, and it extends its use to other applications.

APPARATUS*

The homogenizer is illustrated in Fig. 1. It consists of a plunger, *A*, 8 mm. in diameter, and an enlarged conical tip, *B*, 10 mm. in diameter, which is ground to fit the conical tip, *C*, of the homogenizer tube, *D*. Since the inside diameter of this homogenizer tube is 12 mm., the plunger does not contact the side walls of the tube. If the diameter of the plunger were to approximate the walls of the tube, capillary action would draw the homogenate between these two surfaces, and the fluid loss would be considerably increased. A few milligrams of tissue may be homogenized with 50 μl of fluid in this conical tipped micro homogenizer, and 90 per cent of the fluid can be subsequently removed from the conical tip with a micropipette. Samples of tissue up to 50 mg. may be effectively homogenized.

Use of Conical Tipped Homogenizer in Cell Fractionation Procedures.—In the separation of cell constituents by differential centrifugation, the microhomogenizer can be used to advantage to resuspend the various fractions. When washing cell fractions, vigorous shaking must be avoided, since this denatures protein. The homogenates are centrifuged in the micro homogenizer tubes.† After removing the supernatant, the precipitate is easily resuspended by means of the plunger.

Use of the Micro Homogenizer in Place of Filtration.—Using an adaptation of the Schmidt-Thannhauser method[2] ultramicro separation of ribo and desoxyribo nucleic acid has been carried out on samples of tissues weighing between 2 and 5 milligrams. After homogenizing the tissues in trichloracetic acid they are washed successively in the micro homogenizers with trichloracetic acid, water, organic solvents, etc. The plunger is used to resuspend the precipitate, following which it is lifted part way out of the tube and rinsed. The wash fluid is allowed to flow in the homogenizer tube and the plunger is set aside in the circular rotating rack (Fig. 2) during the subsequent centrifugation.

Use for Micro Refluxing.—In refluxing a precipitate with an organic solvent (e.g., to remove lipids as in the Schmidt-Thannhauser procedure) a teflon rod, T,‡ is placed in the homogenizer (see Fig. 3). This serves as an excellent boiling stick, for it is resistant to organic solvents, acids, and alkali. The homogenizer is attached to a reflux condenser, *F*, by means of a 14/20 standard taper joint, *E*, and is held in place by the nipples, *G*, and the spring, *H*. The tubes are then placed in a water bath and refluxed at the appropriate temperature.

Use of the Micro Homogenizer as a Hydrolysis Vessel.—In the nucleic acid method the precipitates are retained in the homogenizer throughout the various washing and refluxing procedures. The hydrolysis in the M-NaOH is also carried out within the homogenizer.

From the Department of Anatomy, Western Reserve University School of Medicine.
Aided by a grant from the National Foundation for Infantile Paralysis.
Received for publication June 30, 1951.

*Available through the Micro-Metric Instrument Company, Cleveland, Ohio.

†Two rubber cushions, placed in a standard 50 c.c. International Centrifuge cup facilitate the removal of the homogenizer tubes.

‡E. I. DuPont de Nemours & Co., Arlington, N. J.

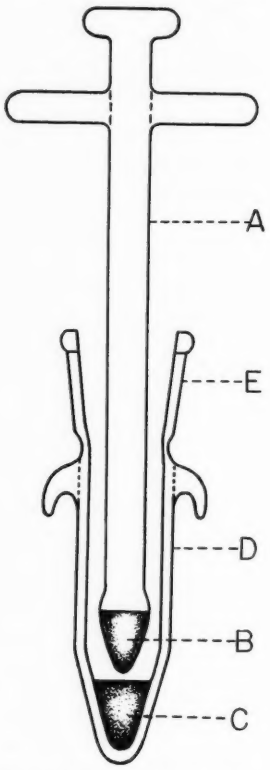

Fig. 1.—Micro-conical tipped homogenizer.

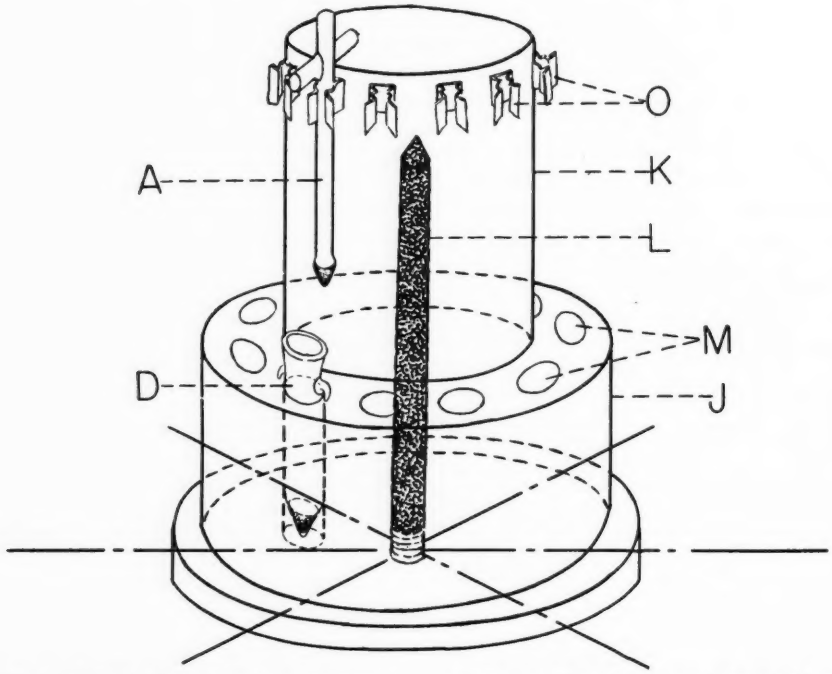

Fig. 2.—Rack for the micro homogenizer. This consists of two cylindrical blocks, *J*, and *K*, which rotate about the vertical rod, *L*. A series of holes, *M*, in the lower cylinder accommodate the homogenizers, *D*, whereas a series of spring clips, *O*, attached to the upper cylinder accommodate the plungers, *A*.

Since a small portion of the precipitate may be adherent to the plunger of the homogenizer, it is desirable to have the tip of the plunger immersed in the hydrolysis fluid (see Fig. 4). A rubber stopper, *P*, is selected to fit the 14/20 standard taper joint. After drilling an 8 mm. hole, a sharp razor cut, *Q*, is made parallel to the long axis of the hole through one-half of the diameter of this stopper. This permits the stopper to be placed about the body of the plunger, *A*, by separating the cut ends and it avoids the necessity of sliding the tip of the plunger through the hole of the stopper. The plunger is positioned as shown in Fig. 4 so

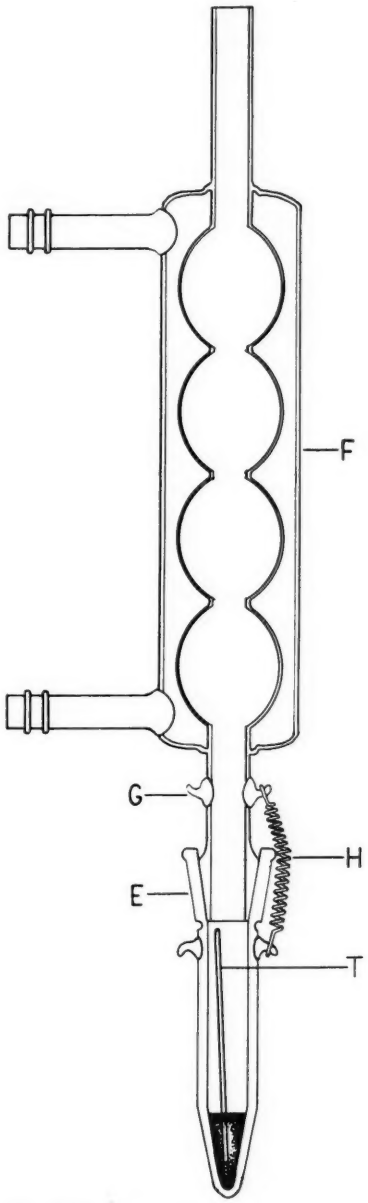

Fig. 3.—Homogenizer tube attached to a reflux condenser.

that it does not quite reach the bottom of the homogenizer. Since the rubber stopper does not provide a perfect airtight seal, the upper portion of the homogenizer, etc., is wrapped with a sheet of parafilm,* S, as shown in Fig. 4. This is especially desirable if the hydrolysis is carried out over many hours.

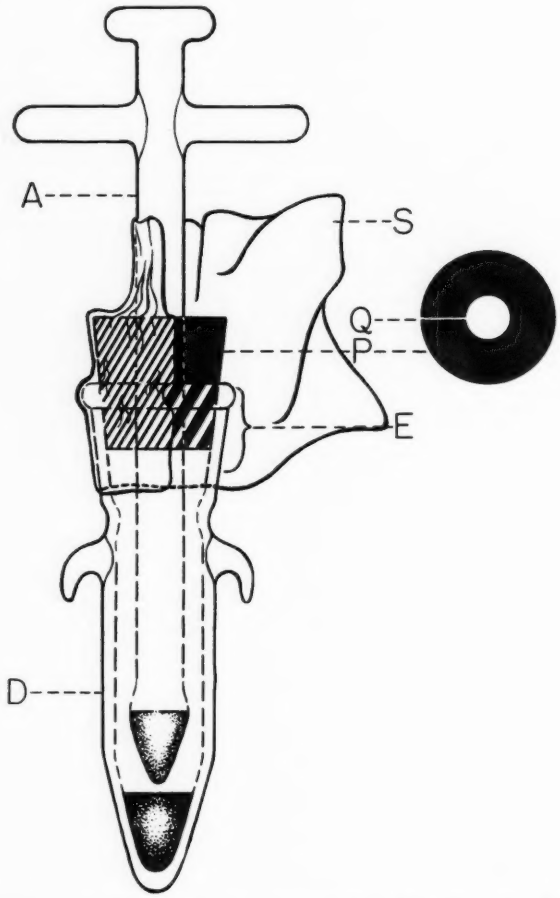

Fig. 4.—Micro homogenizer used for hydrolysis with the plunger inserted through the hole of the stopper. The parafilm sheet, *S*, is shown partly wrapped about the upper end of the homogenizer.

SUMMARY

A simple conical tipped ultramicro homogenizer is described. This apparatus is useful in carrying out a number of microchemical procedures.

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*Marathon Co., Menasha, Wis.

A NEON-TUNGSTEN CUVETTE OXIMETER FOR WHOLE BLOOD

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THE advantages and use of a neon discharge tube as the light source for the photometric determination of the oxygen saturation of hemolyzed blood have been noted previously.[1] The purpose of this investigation was to evaluate the utility of such a light source in an oximeter designed to measure the oxygenation of whole blood, flowing or stationary in the cuvette.

A cuvette oximeter for whole blood designed by Groom and Wood,[2] and similar in principle to the one described by Wood and his co-workers,[3-6] but having neon and argon discharge tubes to provide the red and infrared light sources, was studied. In attempting to calibrate this oximeter it was found that the argon tube (infrared light source) provided an inconstant intensity of light,† and a row of four small 3-volt tungsten filament lamps was therefore substituted for the argon tube. The apparatus finally used consisted of the whole blood cuvette oximeter, or blood cell proper, two galvanometers, a control box in which were mounted the necessary switches and potentiometers, a 2,000-volt transformer, and a 6-volt storage battery. The neon discharge tube (red light source) consisted of clear lead glass tubing with an inside diameter of 2 to 3 mm. and a length of 3.8 cm. The tube was filled with pure neon gas under 20 mm. of mercury pressure, and lighted by 2,000 volts AC. The cuvette consisted of a polythene tube 12.6 cm. in length, having an outside diameter of 2.8 mm. and an inside diameter of 2.0 mm., which was compressed by the brass masking plate to a lumen depth of 0.5 mm. (Fig. 1). The internal volume of the cuvette proper was 0.23 ml. Two photoelectric cells† of the iron-selenium barrier-layer type were used. The photoelectric cell for visible red light was covered by a Wratten 29F filter,‡ while that for infrared light was covered by a double-thickness Wratten 88A filter.§ The area of each cell actually illuminated during use was approximately 1.3 sq. cm.

METHOD OF USE

A detailed description of the principles involved in the use of such an oximeter has been set forth elsewhere.[3-8] The polythene tube of the blood cell filled with saline solution was attached to the arterial needle or cannula. The galvanometer deflections with saline solution in the cuvette were recorded, and then 5 to 10 ml. of blood were drawn at a constant rate through the blood cell and into a heparinized syringe under oil for Van Slyke analysis.[9] During this time galvanometer readings were recorded while blood was flowing and also after the blood was stationary in the cuvette for a period of from thirty to sixty seconds. The cuvette was then flushed out with saline solution until clear of

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†The variability of this light source was apparently caused by shifts in the position of the argon discharge arc within the tube. Attempts to eliminate this characteristic by varying the voltage and frequency of the alternating current power source were unsuccessful.

‡Manufactured by the Bradley Laboratories, New Haven, Conn.

§Manufactured by the Eastman Kodak Company, Rochester, N. Y.

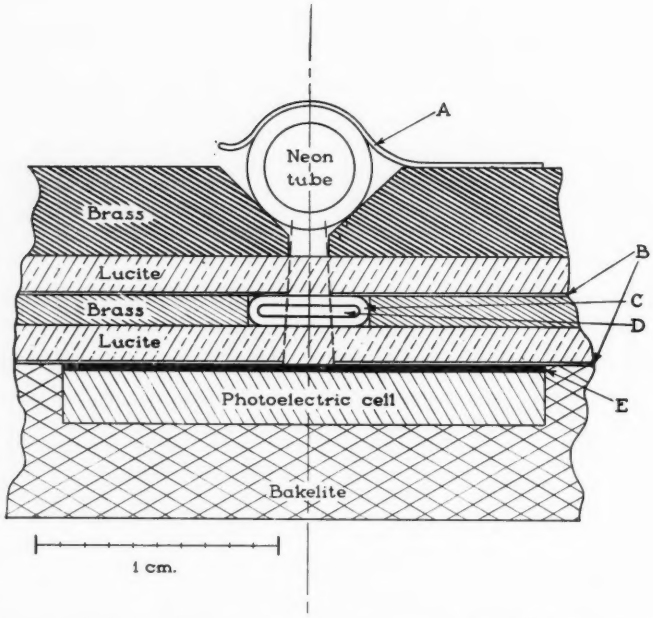

Fig. 1.—Cross-sectional diagram through the neon tube light source of the cuvette "blood cell" to show its relationship to the polythene tube cuvette and the photoelectric cell (drawn to scale). *A*, spring clip; *B*, brass shim stock (0.005 inch) to mask polythene tube; *C*, wall of polythene tube cuvette; *D*, lumen of polythene tube cuvette, through which blood passes, while being transilluminated by the neon and tungsten-filament light sources simultaneously; *E*, Wratten 29F filter.

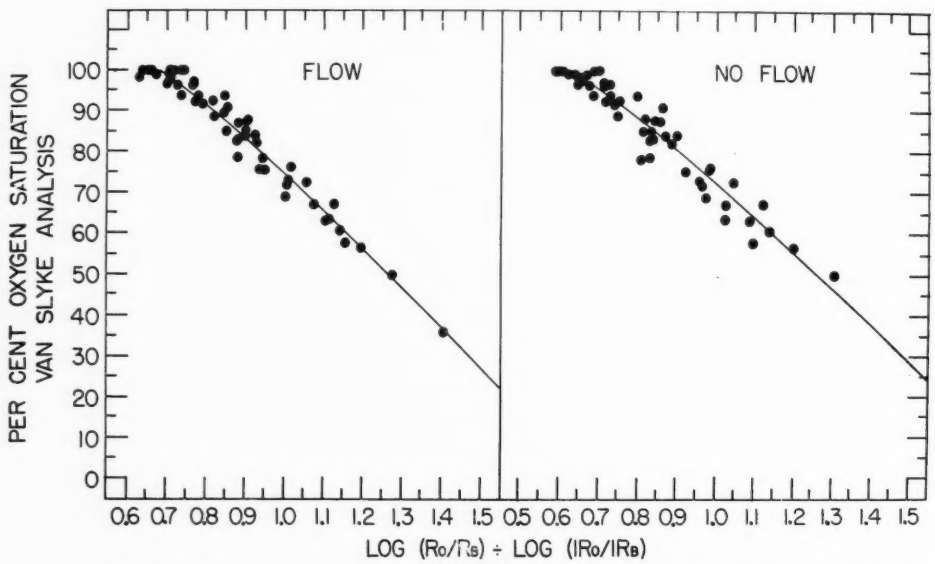

Fig. 2.—Empirical calibration curves of neon-tungsten cuvette oximeter for whole blood flowing or stationary in the cuvette; based on fifty simultaneous photoelectric and Van Slyke determinations of oxygen saturation of both arterial and venous samples of dogs' blood. R_O and IR_O: galvanometer deflections for red and infrared light with saline solution in cuvette. R_B and IR_B: galvanometer deflection for red and infrared light with blood in cuvette.

blood, and the galvanometer deflections with saline solution in the cell were again recorded. The ratios of the optical densities of the blood to visible red and to near infrared light, when flowing and stationary in the cuvette, were calculated as described by Wood and Geraci.[3] Empirical calibration curves were constructed by plotting optical density ratios against the per cent oxygen saturation as determined by manometric analysis.[9]

RESULTS

More than fifty samples of blood ranging in saturation from 36 to 100 per cent were withdrawn through the cuvette from seventeen dogs. The hemoglobin concentration ranged from 11 to 20 Gm. per 100 ml. of blood. The empirical calibration curves are shown in Fig. 2. The accuracy of this method for determining oxygen saturation of hemoglobin was calculated by utilizing the oxygen saturation values determined by the manometric method as a standard.

The standard deviation of the differences of more than fifty simultaneous determinations by the manometric and photometric methods for blood flowing through the cuvette was 2.6 per cent saturation. This implies a 95 per cent likelihood that a single determination made with this instrument will be within 5.2 per cent saturation of the value obtained by Van Slyke analysis. The standard deviation for the photoelectric values obtained when blood was stationary in the cuvette was 3.3 per cent saturation. Allowing for the variability of the manometric method, and assuming that there was no variation in the possible systematic errors of the Van Slyke analysis, the actual standard deviation of the photometric method was calculated to be 2.1 per cent saturation.

It is concluded that the accuracy of this cuvette, incorporating a neon light source, was not superior to that of previously studied cuvettes[4, 5, 10] utilizing tungsten filament bulbs for this purpose.

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NOTE ON APPARATUS FOR DETERMINATION OF URINARY FORMALDEHYDOGENIC CORTICOIDS

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THE purpose of this note is to describe apparatus which we have found useful and convenient in determinations of urinary formaldehydogenic corticoids by the method of Corcoran and Page.[1]

Pipette for Delivery of Chromotrophic Acid Reagent.—This reagent is prepared in 15 M H_2SO_4, so that it is viscous and hard to dispense from usual laboratory equipment. Details of the construction of an automatic pipette for this purpose are shown in Fig. 1. *A* is an inverted 5 ml. pipette, and *B* a chamber to receive overflow from its tip. Detail of *B* is shown in inset B′. *C* is a two-way standard taper, wide-bore stopcock which leads into pipette *A* and, from *A* to the wide (about 1.5 mm. internal diameter) outflow tip which permits rapid delivery of the reagent. Detail of *C* is shown in *C′*. *D* is a reservoir of 150 ml. capacity, made from a standard levelling bulb. A two-way stopcock permits delivery of reagent from *D* through *C* to *A*, or drainage of waste reagent out from the apparatus through delivery tube *E* (detail in *E′*). The necessary rapid delivery of this viscous reagent establishes a 5 ml. calibration at a point closer to *C* than the maker's mark on the pipette.

Apparatus for Distillation of Formaldehyde.—Microdistillation of formaldehyde after periodate oxidation of urinary corticoid has been time consuming and inaccurate in many laboratories. The devises here described facilitate this step in the determination.

Fig. 2 is a photograph of an assembly of seven distillation flasks, condensers, and receivers. The heating units are hemispherical Glascol mantles made to fit 50 c.c. round-bottom flasks. Their power input is regulated from a single powerstat. Details of the distillation flasks, made from heavy, annealed Pyrex glass, and the condenser are shown as *A* and *B*, respectively,* in Fig. 3. *C* is a standard 10 ml. volumetric flask. In practice, 1.5 ml. of water is placed in flask *C*, and the assembly begun by securing flask *A* to condenser *B* by their standard taper joints. Flask *A* contains the reacted mixture of urinary extract, periodate, and stannous chloride. Flask *C* is at first mounted on a wooden block so that the tip of condenser *C* can reach below the water level. After about 6 c.c. of distillate have been collected, the block is removed, and the distillate allowed to drip into the neck of the flask. Power input is set so that distillation is complete (about 8.5 c.c. of distillate collected) at about thirty minutes when the condenser stopcock is opened and the flasks removed. Recoveries of added formaldehyde range close to a mean of 95 per cent.

From the Research Division of the Cleveland Clinic Foundation and the Frank E. Bunts Educational Institute.

Received for publication, July 24, 1951.

*Flasks and condensers can be obtained from the division of Laboratory and Pharmacological Sales Corning Glass Works, Corning, N. Y.

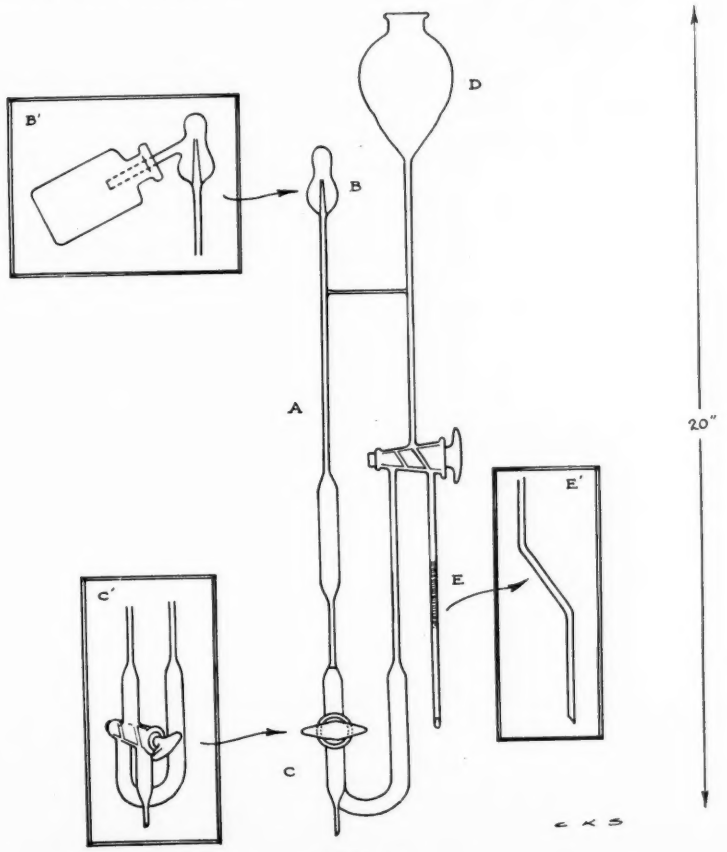

Fig. 1.

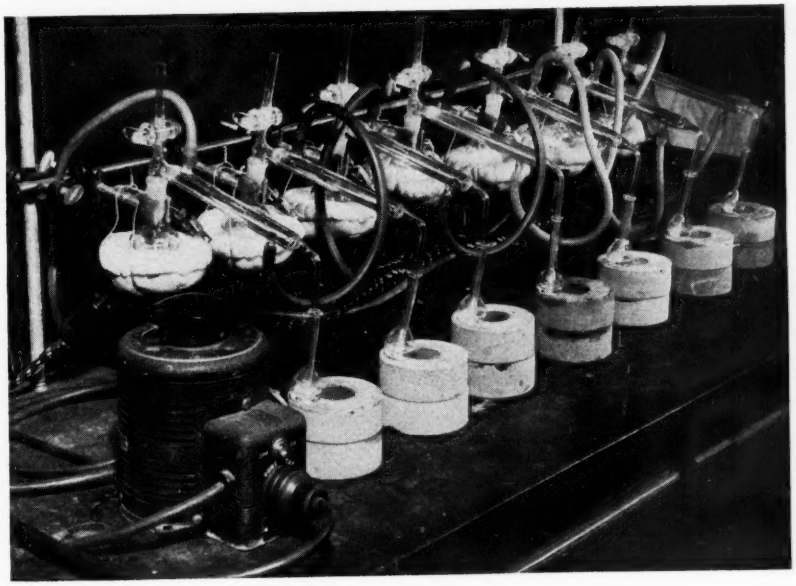

Fig. 2.

SUMMARY

A rapid delivery, automatic pipette for delivery of chromotrophic acid reagent, and a condense assembly for distillation of formaldehyde are described as technical aids to the determination of urinary formaldehydogenic corticoids.

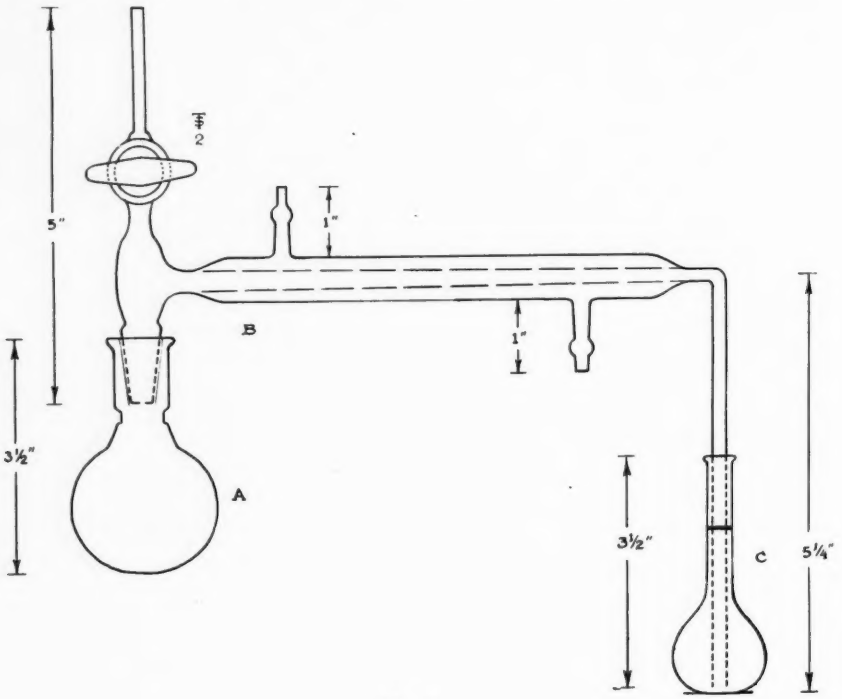

Fig. 3.

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PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Twenty-Fourth Annual Meeting

Chicago, Ill., Nov. 2 and 3, 1951

ABSTRACTS

1. AN EVALUATION OF THE SPATIAL VECTORCARDIOGRAM OF NORMAL YOUNG MEN

J. A. Abildskov, M.D. (By Invitation), G. E. Burch, M.D., J. A. Cronvich, M.S. (By Invitation), and C. E. Jackson, M.D. (By Invitation), New Orleans, La.

Since 1945, several hundred normal and abnormal vectorcardiograms have been obtained in this laboratory using methods previously described. Because of increasing interest in spatial vectorcardiography, this study of seventy-five normal medical students is presented. Plane and stereoscopic projections on the plane surfaces of the equilateral tetrahedron and views sagittal to each were recorded. For correlations with electrocardiography, combinations of simultaneous standard and unipolar leads were obtained. High speed movies, constructed models, and roentgenographic studies were obtained for evaluation of procedures and techniques.

These studies indicate: (a) Sixty-six of the seventy-five normal records could be described as variants of a basic pattern. The average maximal QRS vector measured 0.98 mv. and +66 degrees and 0.86 mv. and +124 degrees in the frontal and sagittal planes, respectively. The average maximal T vector was 0.28 mv. and +47 degrees in the frontal and 0.23 mv. and +124 degrees in the sagittal. Nine records required special consideration, probably because of extreme variations in position, surrounding tissues, or order of electrical events in the myocardium. (b) The equilateral tetrahedron, a spatial extension of the equilateral triangle, is a simple and accurate reference system for recording the spatial vectorcardiogram and permits exploitation of the extensive knowledge available in electrocardiography. (c) The spatial vectorcardiogram will not in its present form define in detail the various electrocardiographic leads. (d) The stereoscopic vectorcardiogram is very informative. Usually a frontal stereoscopic view can replace all other recorded views. Because of man's limited stereoscopic vision, other views are necessary for details of spatial configuration. (e) In no instance did the spatial vectorcardiogram definitely reveal anything of clinical import not indicated by electrocardiography. (f) As currently recorded, the vectorcardiogram fails to reveal such temporal phenomena as duration of intervals and cardiac rhythm. (g) Although the spatial vectorcardiogram failed to be superior clinically to the electrocardiogram in any instance, or to possess any clinical application of its own, it is an interesting and important approach to electrical events in the heart of man warranting experimental use in the laboratory and clinic. Considerable study, experience, and sane evaluation of data must be obtained before clinical applications develop.

2. THE EFFECT OF INTRAVENOUS INJECTION OF THROMBOPLASTIN AND THROMBIN IN DOGS

CLAIR BASINGER, M.D. (BY INVITATION), AND J. GARROTT ALLEN, M.D., CHICAGO, ILL.

Beef lung thromboplastin and bovine thrombin were injected slowly intravenously into two groups of dogs. The injection was completed within forty-five to sixty minutes. These experiments were carried out to determine (a) whether such injections were compatible with life; (b) the effect of these agents upon the clotting mechanism in dogs; and (c) the rate of recovery of the clotting mechanism following such injections.

During the first fifteen minutes of the injection period, whether thromboplastin or thrombin was administered, the animals displayed some dyspnea and restlessness. Thereafter the general reaction was less marked. At this time the systolic blood pressure was decreased and the pulse rate increased. At the end of an hour and a half no further coagulable fibrinogen could be detected in samples. The prothrombin activity was reduced, especially when the agent injected was thromboplastin. The decline in prothrombin was less when thrombin was injected. Thrombocytopenia also occurred. In addition, the fibrinolytic properties of these "plasmas" following injection was increased and may have contributed to the destruction of fibrinogen.

Autopsy sections taken four hours after defibrination disclosed no evidence of fibrin deposits in the lung, liver, kidneys, or spleen.

The reappearance of fibrinogen occurred within two hours, and reached its normal concentration within four to six hours. In some instances fibrinogen returned to above the pretransfusion levels. The rate of prothrombin return to normal concentrations was considerably slower than that of fibrinogen.

Survival was possible, providing the injection was not carried out too rapidly, and the vein at the site of puncture was compressed until fibrinogen reappeared.

It is concluded that intravascular defibrination is possible and compatible with life under these conditions, and the relationship of these observations to the dynamics of coagulation in surgical patients is discussed.

3. HEREDITARY SPHEROCYTOSIS: PATHOGENESIS OF THE "ACUTE CRISIS"

JOHN D. BATTLE, JR., M.D., CLEVELAND, OHIO

(INTRODUCED BY A. C. ERNSTENE, M.D.)

Clinical and hematologic studies of three brothers during concurrent acute crises of hereditary spherocytosis confirm the recent observations of Owren, who noted the presence of temporary marrow aplasia during the acute crisis in six patients. He proposed that "aplastic crisis" replace the term "hemolytic crisis."

The onset was abrupt with headache, malaise, fever, and abdominal pain in each case. The patients were acutely ill with profound pallor, lack of jaundice, and splenomegaly. The crises in the three children developed within a period of eight days. Laboratory studies failed to confirm or deny that infection precipitated the attacks. The history suggested the absence of clinical manifestations of hereditary spherocytosis prior to the acute crisis. Serial blood and marrow studies revealed a profound anemia with a high percentage of microspherocytes, reticulocytopenia, normal icterus index, and hypoplasia or maturation arrest of the marrow during the acute crisis. Ap-

parent spontaneous recovery from crisis occurred without transfusions in a few days, as indicated by a return of the marrow to normal cellularity and maturation, increase of reticulocytes in the peripheral blood, and rapid increase of the hemoglobin, red, and white blood cells. There was no clinical evidence of increased hemolytic activity during the crisis.

Splenectomy was performed in each child during the recovery phase with a similar response observed in chronic active cases.

4. CLINICAL RESULTS AND COMPLICATIONS OF TRIETHYLENE MELAMINE THERAPY IN LEUCEMIA AND ALLIED DISORDERS

EDWIN D. BAYRD, M.D. (BY INVITATION), J. M. STICKNEY, M.D. (BY INVITATION), BYRON E. HALL, M.D., AND CHARLES H. WATKINS, M.D., ROCHESTER, MINN.

Recent reports of the favorable effects of triethylene melamine (TEM) in the treatment of leucemia, malignant lymphoma, and allied disorders led to a therapeutic trial with this substance in forty-three patients. This paper deals with the clinical and hematologic responses, dosage, toxic manifestations, and complications of TEM therapy observed in these patients over a period of approximately one year. The oral route of administration was employed in all cases. Of the forty-three patients, fourteen had chronic lymphocytic leucemia, eight chronic granulocytic leucemia, three acute leucemia, seven Hodgkin's disease, seven lymphosarcoma of various types, two myeloma, one neuroblastoma, and one ependymoma.

The disease responding most favorably to TEM therapy was chronic granulocytic leucemia. Good but less uniform responses were observed among patients having chronic lymphocytic leucemia, Hodgkin's disease, lymphocytic lymphosarcoma, and giant follicle lymphoma. Patients having acute leucemia, myeloma, and carcinoma were not benefited. In general, responses to treatment varied widely, even among patients having the same disease.

Toxic manifestations included anorexia and nausea which lasted twelve to twenty-four hours after ingestion of the drug, hypoplasia of the bone marrow, thrombocytopenia accompanied by hemorrhagic phenomena, and pathologic changes in the glomeruli of the kidneys. Thrombocytopenia and hemorrhage proved to be a serious complication in a number of patients with chronic lymphocytic leucemia, necessitating multiple transfusions. One patient who experienced thrombocytopenia while on maintenance therapy died from cerebral hemorrhage. On the other hand, thrombocytopenia was not observed among patients having chronic granulocytic leucemia.

5. STUDY OF HEMODYNAMICS IN COARCTATION OF THE AORTA WITH USE OF DYE DILUTION AND DIRECT INTRA-ARTERIAL PRESSURE RECORDING METHODS*

EARL F. BEARD, M.D. (BY INVITATION), HOWARD B. BURCHELL, M.D., EARL H. WOOD, PH.D., AND O. THERON CLAGETT, M.D. (BY INVITATION), ROCHESTER, MINN.

The Stewart-Hamilton dye injection method, if used with T-1824 and cuvette oximeter-strain gauge manometer assemblies attached to indwelling arterial needles as detecting devices, allows estimation in one experiment of (a) circulation times, (b) cardiac output, (c) total blood volume, (d)

quantity of circulating blood between injection and sampling points, and (e) data from intra-arterial pressure pulse tracings. Time-concentration curves of dye, following instantaneous intravenous injection, were recorded at the ear (by ear oximeter), radial artery, and femoral artery in five normal subjects, preoperatively in fourteen patients with coarctation of the aorta, and again in six patients after surgical resection of the stenosis.

Contours of dye curves recorded at all three points in patients before and after surgery were qualitatively similar to those recorded in normal subjects (unlike curves produced by intracardiac shunt). Objectively measured circulation times to the ear and radial artery in patients with coarctation were only slightly less than in normal subjects, while those to the femoral artery were normal. Surgery slightly increased circulation times to ear and radial artery, while those to the femoral artery were unchanged. There was no significant difference between average cardiac index values in normal subjects (3.9 ± 0.3*), preoperative coarctation patients (4.0 ± 0.2), and postoperative patients (3.5 ± 0.3 liters per minute per square meter). However, comparison of cardiac index values in the same six patients before and after surgery revealed a significant postoperative decrease, averaging 0.5 ± 0.1. No significant differences in total blood volume were found among the three groups of subjects by the method used. In normal subjects the estimated volume of actively circulating blood between injection point and radial artery was slightly greater than that between injection point and femoral artery (average difference of 0.4 ± 0.1 liters). These two quantities were almost identical in preoperative coarctation patients, but postoperative patients showed the relationship found in normal subjects. Alterations in intraradial pressures, intrafemoral pressures, and conduction times of the femoral pulse were in agreement with previous studies, as was the return toward normal of these variables postoperatively.

6. LACK OF SELECTIVE UPTAKE OF RADIOACTIVE IODINE PHOSPHORUS, AND COPPER BY MELANOMAS IN MOUSE AND MAN

WILLIAM H. BEIERWALTES, M.D., AND C. T. KNORPP, M.D. (BY INVITATION), ANN ARBOR, MICH.

The epidermal melanoblast utilizes tyrosine as a substrate in the elaboration of melanin. The metabolism of I^{131} tagged tyrosine may be similar to untagged tyrosine in the melanoblast. The enzyme tyrosinase is also essential in the elaboration of melanin. This is a copper-protein complex. It seemed reasonable to supply radioactive copper, Cu^{64}, as a possible substitute for stable copper ordinarily present in this enzyme. Radioactive phosphorus, P^{32}, might be supplied on the basis of an increased uptake for nuclear metabolism in rapidly growing melanomas.

Nine radioisotope studies were made on six human beings with malignant melanomas. Each patient was given 1 mc. of I^{131} orally. Counts 100 seconds in duration were made over the tumor on one extremity and then over the same anatomy on the opposite extremity, using a similar geometry, with a shielded localizing counter immediately after the dose, then at ten-minute intervals to one hour, twenty minutes to two hours, then every two hours to eight hours, and again at twenty-four hours. Two of these patients were then given 1 mc. of P^{32} orally, and the same procedure followed to twenty-four hours. One patient was then given 500 μc of Cu^{64} in a total of 13.6 mg. of stable copper intravenously and followed in a similar manner for twenty-

*Figures following ± signs are standard errors of the means.

four hours, with additional counts over liver and spleen. Uptake count values followed a characteristic curve for each isotope, but showed no selective concentration of any of the isotopes by the melanomas.

A similar study was performed fourteen times on seven mice. Both Hardman-Passey and Cloudman strains of melanoma-bearing mice were injected intraperitoneally with 100 μc of I^{131} in 0.2 c.c. of distilled water, or the same quantity of P^{32}, or 64 μc of Cu^{64} with 24 mg. of stable copper, and showed no selective concentration of these isotopes in the tumors by in vivo or in vitro counting.

7. AN EVALUATION OF THE EFFECTS OF CORTISONE ON THE SUBCUTANEOUS NODULES OF PATIENTS WITH RHEUMATOID ARTHRITIS

GRANVILLE A. BENNETT, M.D., SIMON ZIVIN, M.D. (BY INVITATION), IRVING E. STECK, M.D. (BY INVITATION), AND MAX M. MONTGOMERY, M.D. (BY INVITATION), CHICAGO, ILL.

Six patients with rheumatoid arthritis were selected for study. Each patient had paired, symmetrically placed subcutaneous nodules, and in this study eight nodules on one upper extremity were removed prior to the injection of cortisone, and eight were examined after a period of cortisone administration.

The physical characteristics of all nodules were recorded. After the removal of one of each pair of nodules in five of the six patients, the area enclosing the remaining nodule was encased in a plaster cast; the resulting molds served as permanent records of the size and configuration of the nodules. Cortisone was then given. At the end of the observation periods, the size, shape, and consistency of the nodule was determined, and another impression of the nodule was recorded in a plaster mold in each of the same five patients. The nodule was then excised for microscopic examination.

Daily intramuscular injections of 100 mg. of cortisone were administered for eight days to one patient, for twenty days to three, for twenty-two days to one, and for twenty-six days to one. The impression was held that all nodules became softer during administration of cortisone. In four of the five instances in which comparative volumetric measurements were made utilizing the mold impressions, the nodules had diminished in volume (14 to 56 per cent); the fifth had increased 5 per cent. The three remaining nodules, measured dimensionally, had decreased slightly in size.

Microscopic studies comparing the histologic features of each of the eight pair of nodules revealed no consistent qualitative differences in encapsulation, inflammation, palisading of cells, or necrosis that could be attributed to cortisone administration over the period of time selected for this study. The degree of activity in three nodules removed after cortisone injection appeared the same as in the paired nodules excised prior to cortisone administration. In the remaining five instances, the degree of cellular activity and necrosis was greater in the specimens removed after cortisone had been given than it was in the paired nodules removed before cortisone was administered.

8. RENAL FUNCTION IN DIABETIC COMA: LOWER NEPHRON NEPHROSIS WITHOUT OLIGURIA

LIONEL BERNSTEIN, M.D. (BY INVITATION), EDMUND F. FOLEY, M.D., AND WILLIAM S. HOFFMAN, M.D., CHICAGO, ILL.

In four of six patients studied with specific renal function tests and electrolyte clearances during diabetic coma and after recovery, there was depression of some or all specific renal functions during coma attributable to temporary exclusion of a portion of the nephrons because of dehydration. These decreases in function were readily reversed by correction of dehydration and electrolyte deficit. Elevated serum nonprotein nitrogen levels were reduced to normal within forty-eight hours. Both during coma and later, sodium excretion paralleled that of PAH, indicating that those nephrons functioning during coma did so in a normal manner. The amounts of sodium and PAH excreted during coma were less than those following recovery because of participation of fewer nephrons.

In contrast serum nonprotein nitrogen of the other two patients increased for several days in spite of correction of dehydration, reaching peaks of 158 and 122 mg. per 100 c.c., and subsiding to normal after two weeks. This indicated a renal origin of azotemia. Specific renal function tests were all low during coma and only slowly increased in subsequent experiments. In the first experiment C_M/C_{PAH}, C_M/Tm_{PAH}, and C_{PAH}/Tm_{PAH} were all abnormally high. These findings with subsequent slow return to normal constitute the described pattern of lower nephron nephrosis. At the early recovery stage, sodium excretion paralleled that of chloride rather than that of PAH because of nonspecific diffusion of PAH across the lower nephron cells. The absence of oliguria after the correction of the initial dehydration and shocklike state does not exclude the diagnosis of lower nephron nephrosis. Oliguria is produced when nonspecific resorption is great in relation to glomerular filtration. Severe azotemia can still occur when the output is 1,000 to 2,000 c.c. of poorly concentrated urine, especially during acute protein breakdown.

9. AN OBJECTIVE METHOD OF DRUG EVALUATION IN CORONARY ARTERY INSUFFICIENCY RESULTS WITH KHELLIN

MAURICE M. BEST, M.D., LOUISVILLE, KY.

(INTRODUCED BY J. MURRAY KINSMAN, M.D.)

The production of relative anoxemia of the myocardium by means of exercise or the inhalation of a 10 per cent oxygen-nitrogen mixture is often accompanied by abnormal electrocardiographic findings in the presence of coronary artery disease. The Master "two-step" test and the anoxemia test of Levy have been accepted as useful aids in the diagnosis of angina pectoris. We have found these procedures useful as an objective means of study of drug efficiency and the establishment of an optimal dosage in patients with coronary artery insufficiency.

Eleven patients were studied for a control period of one year. The "two-step" and anoxemia tests were conducted monthly and remained constantly positive. The amount of exercise and duration of inhalation of the reduced oxygen mixture necessary to cause pain and a positive electrocardiographic response was recorded following each test. These remained fairly constant for each patient during the control period. These patients were then used in the drug evaluation. Both tests were then repeated at the conclusion of each eighteen-day testing period. The treatment periods consisted of the oral

administration of a crude Khellin, 40 mg. three times a day, and a crystalline Khellin (from which the visnagin had been removed) in dosages of 10, 20, 30, and 40 mg. three times a day. These periods were alternated with placebo periods of similar length. Conclusions were made on the basis of the ability of the drug to delay or prevent pain and abnormal electrocardiographic responses in a tolerated dosage.

The data suggest that Khellin is often effective in the reduction of the relative myocardial anoxemia induced by exercise or inhalation of a reduced oxygen mixture. The drug may be effective in doses which are smaller than heretofore recommended. Toxic effects are reduced somewhat by the elimination of the visnagin component, but they continue to limit the clinical usefulness of the drug.

10. WHITE BLOOD CELL COUNTS IN THE LEUCEMIAS

WILLIAM R. BEST, M.D. (BY INVITATION), LOUIS R. LIMARZI, M.D., AND HENRY G. PONCHER, M.D., CHICAGO, ILL.

Characterization of the white blood cell count at initial observation in the leucemias is usually stated very generally with notations on extreme range. Calculations of mean and standard deviation for a given type of leucemia are meaningless due to the marked skewness of distribution. Due to this skewness and to the known exponential behavior of many biological phenomena, the logarithm of the white blood cell count is analyzed here rather than the count itself. In 149 cases of chronic lymphatic leucemia the arithmetic "mean" white blood cell count was 125.3 (in each case figures indicate thousands per cubic millimeter) with only 20 per cent of observations being greater. The mean by logarithmic analysis was 53.6 which compares closely with the median of 53.1. The one standard deviation limits ordinarily encompass approximately the central two-thirds of observations. These limits derived logarithmically were 14.2 and 200.0, as compared with limits for lower and upper sixths of 14.0 and 226.0. Similar comparison of logarithmic with percentile data for the other types of leucemia showed almost comparable agreement. Logarithmically derived mean and one standard deviation limits in ninety-eight chronic phase, chronic myeloid leucemias were 104.0 (25.3-424); twenty-six acute exacerbation, chronic myeloids were 30.7 (6.7-223); fifty-seven acute stem cells, 13.0 (2.9-58); seventy-two acute myeloids, 16.0 (3.3-78.5); twenty-nine leucemic reticulo-endothelioses, 5.0 (1.3-20.8); and twenty-nine monocytics, 26.0 (11.1-61.4).

When the individual data are graphed logarithmically, a normal distribution is noted with chronic lymphatic leucemias, some skewness to the right with chronic myeloid, and small secondary peaks in the higher ranges of the acute leucemias. Thus, logarithmic analysis of white blood cell counts permits more accurate characterization of each leucemic type. The pattern of distribution for each variety may ultimately be correlated with pathogenic mechanisms.

11. LONGEVITY IN LEUCEMIA

WILLIAM R. BEST, M.D. (BY INVITATION), LOUIS R. LIMARZI, M.D., AND HENRY G. PONCHER, M.D., CHICAGO, ILL.

The characteristic duration of illness from first symptom to death in the leucemias is not accurately represented by mean longevity. In each large series of a leucemic type, a few patients will live much longer than is usual, inordinately influencing the calculated mean. Reports frequently originate

from active services in which many patients may yet be alive. Their inclusion in the analysis may make longevity appear shorter than actual as their disease is still in its course; their exclusion would often rob the statistics of cases with unusually long survival. A method of accumulative analysis on a logarithmic time scale was developed to incorporate incomplete and unusual data in the statistics, at the same time giving a true representation of longevity trends. Percentage survival in each longevity interval is calculated, not on the basis of all cases studied, but on the basis of all cases known dead plus those alive and surviving at least to the lower limits of that interval. When plotted on semi-logarithmic paper, longevity times may be determined for any given percentage of survival. Cases herein analyzed were observed over a fifteen-year period. Chronic leucemias were treated variously with roentgen irradiation, urethane, blood transfusions, and/or other commonly employed agents. The acute and miscellaneous leucemias received supportive therapy but no anti-folic acid drugs. Ninety per cent, *50 per cent,* and 10 per cent of 119 chronic lymphatic leucemias survived at least for 0.8, *2.9,* and 7.8 years, respectively. Similar percentages of ninety-eight chronic myeloid leucemias survived at least for 0.6, *2.4,* and 5.8 years; of forty-three acute stem cells, for 1.6, *3.7,* and 9.2 months; of sixty-three acute myeloids, for 1.2, *3.4,* and 8.0 months; of twenty-two leucemic reticulo-endothelioses, for 1.4, *3.9,* and 15.4 months; and of twenty-five monocytics for 0.6, *3.5,* and 25.0 months. One- and five-year survival rates, respectively, in chronic lymphatic leucemia were 85 per cent and 20 per cent; in chronic myeloid leucemia, 79 per cent and 13 per cent. One-half and one-year survival rates, respectively, in acute stem cell leucemia were 26 per cent and 5 per cent; in acute myeloid, 19 per cent and 5 per cent; in leucemic reticulo-endotheliosis, 31 per cent and 16 per cent, and in monocytic leucemia, 30 per cent and 20 per cent.

12. CLINICAL AND STATISTICAL EVALUATION OF FOUR-HOUR EOSINOPHIL RESPONSE TESTS

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CHICAGO, ILL.

There has been widespread use of the four-hour eosinophil response test to corticotropin (Thorn test), epinephrine, and ephedrine in an effort to assess the status of adrenal, pituitary, and hypothalamus, and for the diagnosis of Addison's disease and hypopituitarism.

To evaluate the clinical usefulness of these procedures 284 miscellaneous medical subjects were repeatedly tested with these agents and with placebos for a total of 702 tests. The standard deviation of repeated ephedrine response tests in ninety-five subjects was 22 per cent. This variation was greater in subjects with low initial eosinophil counts (below 100 per cubic millimeter) and in those with little or no eosinopenic tendency. Second tests to an agent tend to show a diminished response if performed within three days of the previous ones.

Mean eosinopenic response to placebo was 10 per cent, to ephedrine was 41 per cent, and to corticotropin was 62 per cent. These mean differences are statistically significant, but there is a wide overlap of individual observations. One-sixth of non-Addisonian subjects show greater than 50 per cent eosinopenia to placebos, whereas another one-sixth show less than 40 per cent eosinopenia to corticotropin. Most of these latter showed adequate response on retesting; initial low values reflecting the variability of the test. Other

failures were attributed to inactive preparations and testing in the midst of "alarm" reactions. Two Addisonians had less than 20 per cent eosinopenia on repeated tests to these agents. Greater than 50 per cent response to ephedrine was seen in some subjects with anorexia nervosa, carcinomatous cachexia, and pituitary tumors.

We conclude: (a) single tests to corticotropin, epinephrine, or ephedrine which fail to show significant eosinopenia do not establish the diagnosis of Addison's disease; (b) marked eosinopenia with these tests indicates adequate adrenocortical function if initial count is high enough to rule out intrinsic errors; (c) repeated failure of response to ephedrine or epinephrine cannot be construed as indicating organic disease of adrenal, pituitary, or hypothalamus; (d) repeated failure of corticotropin to produce significant eosinopenia is strong evidence for nonfunctioning adrenals, and (e) the level of response to epinephrine or ephedrine does not appear to be an accurate index of pituitary-adrenal function.

13. THE USE OF MEPHENESIN IN CONTROLLING THE SPASMS OF TETANUS

J. Park Biehl, M.D., and Robert A. Helm, M.D., Cincinnati, Ohio

(Introduced by Morton Hamburger, M.D.)

While the significance of the various factors entering into the mechanism of death in tetanus is not universally agreed upon, there is no doubt that muscular relaxation is desirable during treatment. An ideal drug for this purpose would, in addition to relieving spasms, have no inhibitory effects upon the respiratory center, allowing the patient to breathe and cough as required, and would provide only mild sedation.

Mephenesin was chosen as a drug having these qualities. It acts upon the internuncial cells of the brain stem and cord, with little or no effect on the medullary vital centers or cortical synapses except in much higher concentrations. Its use intravenously in single doses has been described repeatedly without mishap.

Six cases of tetanus have been treated on the medical service of the Cincinnati General Hospital with mephenesin administered by intermittent intravenous drip. In addition, there were the usual measures of providing a private quite room with a special intern or nurse, surgical débridement, antitoxin, tracheotomy, and oxygen. Sedation was occasionally added, but not to the point of anesthesia except at times when mephenesin appeared to be giving inadequate results. Three of the six patients survived.

In each case, striking effectiveness in relieving trismus, muscular rigidity, and opisthotonic spasms was observed. This was accomplished with only minimal sedative effects, and with no inhibition of coughing. Subjective improvement was at times dramatic. If more severe generalized convulsions occurred, however, pentothal given to the point of narcosis was usually necessary. In each of the three fatal cases, death occurred at a time when the spasms had been well controlled for hours.

No undesirable effects upon blood pressure, pulse, respirations, or upon the state of consciousness were observed. Slight intravascular hemolysis was suspected in one case, but no harmful effects of this were observed in the patient.

Mephenesin is a useful adjunct in the management of tetanus. While an improvement in the rate of recovery cannot be demonstrated at this time, continued use of the drug seems to be recommended.

14. BIOLOGIC STUDIES WITH ARSENIC[76]
IV. THE HISTOPATHOLOGIC EFFECTS OF ARSENIC[76] IN PATIENTS WITH LEUCEMIA AND RELATED DISEASES

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A total of eighty-two biopsies from the liver, spleen, nodes, and marrow of eighteen patients with neoplastic hematopoietic diseases before and after treatment with radioactive arsenic (As^{76} were studied. Remissions were in general similar to those following P^{32}.

Comparison of the biopsies emphasized the absolute necessity of obtaining adequate control tissues immediately prior to treatment before ascribing any change to As^{76} rather than to variations inherent in the disease. Marked variations in the number of mitoses and in amount of cellular debris were found in both control and post-treatment biopsies in the various patients and even in the same patient. This was particularly true of a case of metastatic carcinoma where no tumor was found in two of five hepatic biopsies.

In general, the changes detected after treatment were slight, consisting of increased nuclear debris and decreased cellularity of the tissues, and in no way corresponded in degree to some of the remissions obtained. Occasionally excellent remissions were obtained without any histologic evidence of an effect ascribable to As^{76}. In both cases of subacute leucemia where serial liver biopsies had been performed, a clear-cut decrease in cellularity of the portal leucemic infiltrate was demonstrable.

There was no evidence that irradiation exerted a selective effect upon immature, rapidly dividing cells. The reticular cell, the most immature cell in the hematopoietic tissues, did not show any evidence of treatment with As^{76}. There was no evidence in the tissues, particularly in the spleens of patients with chronic myelogenous leucemia, that irradiation changed the ratio of immature to mature cells, even in patients undergoing a remission as regards physical findings and peripheral blood counts. In no case studied was there any change in the abnormal pattern of tissue architecture which is characteristic of the leucemias and multiple myelomas.

In general, the effects of AS^{76} upon the tissues were similar to those seen after treatment with P^{32} and nitrogen mustard, and suggest the inability of these cytocidal techniques to effect any fundamental change in the hematopoietic tissues of patients with leucemia.

15. PULMONARY HEMODYNAMICS IN CHRONIC PULMONARY DISEASE

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(INTRODUCED BY JOSEPH H. HOLMES, M.D.)

Twenty-five patients with chronic pulmonary disease have been studied in an attempt to determine the effect of their disease upon the pulmonary circulation. This study included pulmonary and circulatory function tests. Pulmonary function studies included: measurement of the maximum breathing capacity, determination of the total lung volume and its subdivisions, performance of exercise tolerance on the treadmill, and determination of total blood and plasma volumes. Pulmonary circulatory function was evaluated by means of venous cardiac catheterization, and included cardiac indices at rest and with exercise, and peripheral arterial and pulmonary arterial pres-

sures at rest and with exercise. Total pulmonary vascular resistance was determined in all patients. Pulmonary capillary pressures were determined in a number of these patients. Peripheral arterial oxygen saturation levels were measured both at rest and with exercise. The effect of inhalation of 100 per cent oxygen on pulmonary artery pressure and upon arterial oxygen saturation level was determined in a limited number of cases.

These studies revealed that the mean resting cardiac index was a low normal and that with exercise there was but a slight increment. The resting pulmonary artery pressure was considerably elevated in all patients, and there was a further rise on exercise. The total pulmonary artery resistance was elevated, and in a limited number of patients with capillary pressure determinations, the data suggested that the pulmonary arteriolar resistance was responsible for the over-all elevation.

The resting level of the oxygen saturation of the peripheral arterial blood was considerably decreased, the mean being 78 per cent. There was no significant change with exercise. There was no apparent correlation between the peripheral arterial oxygen saturation level and the pulmonary arterial pressure. Likewise, there was no apparent correlation between the peripheral arterial oxygen saturation level and the cardiac output. The inhalation of 100 per cent oxygen resulted in an apparent significant decrease in the mean pulmonary arterial pressure in ten patients so studied.

16. STUDIES ON THE LEUCOCYTIC CHANGES DURING THE TUBERCULIN REACTION

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(INTRODUCED BY CLAUDE-STARR WRIGHT, M.D.)

The leucocytic changes in tuberculosis, particularly in the lymphocytes and monocytes, have been the subject of much investigation. Sabin, Doan, Cunningham, and Wiseman have established the importance of the monocyte-lymphocyte ratio in prognosis. In progressive tuberculosis the ratio is increased, while healing lesions are accompanied by a decreased ratio. Our attention has been focused upon the mechanism of the lymphocyte decrease in progressive tuberculosis.

A study in fifty-two tuberculous and twenty normal rabbits showed that the intravenous and intracutaneous injection of purified protein derivative of tubercle bacilli into the tuberculous rabbits resulted in a 70 per cent decrease in the absolute number of circulating lymphocytes. Normal rabbits had only a slight decrease. This lymphocytic decrease was progressive beginning in one hour and reaching a maximum in six to nine hours, after which the count returned to normal. The reaction was independent of the diurnal variation. This lymphocytic reaction of tuberculous rabbits to P.P.D. resembles that observed by White and Dougherty following the injection of adrenal cortical hormones into normal animals. However, no evidence was obtained to indicate that adrenal cortical stimulation was the mechanism of the lymphocytic decrease during the tuberculin reaction. It is thought that the lymphocytes, being hypersensitive, are lysed in vivo by P.P.D. in a manner analogous to that observed in vitro by Favour. The lymphocytic reaction of tuberculous animals to P.P.D. was dependent upon the hypersensitive state, since rabbits sensitized with dead tubercle bacilli react in a manner similar to the tuberculous rabbits. It is suggested that this lymphocytic response to P.P.D. may be the cause of the observed lymphopenia in progressive tuberculosis, and that it may play a role in the delayed cutaneous reaction.

17. EFFECTS AND CLINICAL SIGNIFICANCE OF BACTERIAL CONTAMINANTS IN TRANSFUSED BLOOD

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Most febrile transfusion reactions are nonhemolytic and occur with disposable equipment allegedly free of pyrogens. To determine if bacterial contamination may produce these nonhemolytic febrile reactions, 1,697 consecutive pints of blood were cultured before leaving the bank. Contaminants were isolated from thirty-eight (2.24 per cent). Heavy growth by diphtheroids in one bottle produced the only febrile reaction. In the remaining thirty-seven, bacteria numbered fourteen colonies per milliliter or less, and no reactions resulted. Organisms most frequently isolated were staphylococci.

The low rate of reaction from contaminated bloods was explained by studying growth curves of ninety-three organisms and bactericidal action of 162 bloods. Eighty-four bloods, inoculated with 200 bacteria per milliliter or less, killed or suppressed various contaminants at room temperature. In seventy-eight bloods manifesting heavy growth in twenty-four hours, the lag phase preceding multiplication always exceeded six hours, a period longer than required for giving transfusions. Twenty-seven inoculated bloods retained bactericidal power while refrigerated for seven weeks. In three others, killing power disappeared in four weeks; subsequently, heavy growth developed in the refrigerator within one week. Bactericidal action depended on a heat labile factor not restored by guinea pig complement.

Potential danger to recipients of blood with heavy bacterial growth was investigated in vitro and in rabbits. Plasma hemoglobin levels higher than 100 mg. per cent were caused in vitro by eight of thirty-one strains, while eight nonhemolytic strains distinctly increased erythrocyte fragility. Numerous strains of fourteen species clotted blood by consuming citrate. Blood type was altered only once in 113 contaminated specimens. (O to AB by *Ps. aeruginosa.*)

Rabbits transfused with heavily contaminated blood containing staphylococci experienced fever but no serious harm. By comparison, *A. aerogenes* was equally pyrogenic, but usually produced shock and death, caused intravascular hemolysis, and was eliminated less rapidly from the blood stream than staphylococci. Diphtheroids were consistently pyrogenic, but of variable toxicity.

It is probable that the demonstrated factors which protect recipients from the potential harmful effects of contaminated blood may be overcome if sufficient incubation occurs at room temperature or in the refrigerator, and especially if the inoculum is large.

18. THERAPY OF MALIGNANT DISEASES WITH PROTEINAMINE (A NEW AGENT)

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(INTRODUCED BY BEN E. GOODRICH M.D.)

Under certain conditions methyl-bis-(betachloroethyl)-amine couples with plasma proteins to form products having radiomimetic activity. Kelly, Mangun, Sanders, and Hartman have described these agents and have labelled

them collectively as proteinamine. This study is concerned with the use of proteinamine substances intravenously in the treatment of malignant disease.

Fifty-nine patients have been given a total of more than 600 doses of proteinamine. 97.8 per cent of injections were given without producing symptoms other than mild anorexia. Neither agranulocytosis nor severe thrombocytopenia was encountered.

Clinical improvement was obtained on one or more occasions in seven of twelve cases of Hodgkin's disease, eight of which were far advanced. Favorable clinical responses occurred in three of four cases of lymphosarcoma; in two of seven cases of acute lymphatic leucemia; and in two of four cases of acute monocytic leucemia. Repeated remissions occurred in one of two cases of lymphoepithelioma. One case each of Ewing's sarcoma, neuroblastoma, hemangiosarcoma, and embryonal-cell carcinoma responded well to therapy. Remissions were induced in two cases of polyarteritis and one of two cases of acute disseminated lupus erythematosus. There was no improvement in one case of acute myelogenous leucemia, one of chorioepithelioma, and two of multiple myeloma. Transient symptomatic benefit was observed in four of thirteen cases of metastatic carcinoma.

The authors feel that the results of treatment are comparable to those available with nitrogen mustard and that proteinamine possesses certain advantages: (a) virtual absence of nausea and vomiting; (b) reduced hematopoietic toxicity; (c) children and patients with acute leucemia tolerate proteinamine; (d) suitability for use in outpatients.

19. A PRELIMINARY REPORT ON THE EFFECT OF CERTAIN 8-AMINOQUINOLINES IN THE TREATMENT OF RHEUMATOID ARTHRITIS

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The clinical response of thirty-eight patients with rheumatoid arthritis to treatment with primaquin and three chemically related 8-aminoquinolines has been studied. Patients were classified in accordance with "The Criteria for Therapeutic Response of Rheumatoid Activity" of the American Rheumatism Association. The patients studied consisted of seventeen women and eighteen men in stages II and III of rheumatoid arthritis, active, with functional capacity of Class II to Class IV. Three men with rheumatoid spondylitis were also treated.

Clinical records of thirty-three of the patients who had previously been followed for long periods are available to us. Patients were hospitalized for six weeks, consisting of two weeks evaluation, two weeks of oral drug administration, and two weeks post-treatment observation. Routinely complete blood counts, erythrocyte sedimentation rates, plasma protein studies, and roentgenograms of involved joints were done. Photographs, motion pictures, and joint measurements were taken before and after medication in many cases. Placebos were used initially for some of the patients. No corrective procedures of any sort were permitted except limitation of caloric intake for obese patients.

During the course of therapy twenty patients have made response of Grade II or better, fifteen patients minor or no improvement. The three spondylitics have responded well. Several patients continue in remission

months after drug was stopped, but most of them tend to relapse within two to three weeks. Many of these are currently being followed as outpatients receiving small amounts of test drug two or three days a week.

Toxic manifestations include methemoglobinemia, anorexia, abdominal distress, and neutropenia. The white count returned to normal when drug was discontinued.

The present study suggests that the compounds possess some degree of antirheumatic activity. Further exploration of the 8-aminoquinoline group of compounds in a search for a drug of greater therapeutic potency and less toxicity seems warranted.

20. STUDIES IN THE NATURE OF THE DEEP K STROKE BALLISTOCARDIOGRAM

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The form of the ballistocardiogram in which the K stroke predominates the complex in duration and depth, and termed the "deep K stroke pattern," is considered an abnormal form. Its appearance in cases of hypertensive cardiovascular disease has been noted especially. The present study was undertaken for evaluation of the significance and genesis of this pattern.

A portable electromagnetic ballistocardiograph machine, with a resistance-coupled amplifier, was used with photographic recording. The appearance of the deep K stroke pattern in normal individuals was noted. The effect of sympathicolytic and hypotensive ergot preparations on the B.C.G. form of hypertensive individuals with pre-existing deep K stroke pattern was studied. Conversely, an analysis of the changes produced in the B.C.G. form in normal individuals during blood pressure elevation by 1-nor-epinephrine was made. The significance of the observed changes is discussed.

21. PROTECTION AGAINST HYPERTENSION, ARTERIOLONECROSIS, AND DEATH BY HOG RENAL EXTRACTS IN EXPERIMENTAL MALIGNANT HYPERTENSION

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Hog crude renal cortex extract containing renin was highly effective in protecting against the hypertension of experimental malignant renal hypertension in dogs when the antirenin titer was 2.5 AU (antirenin units) per cubic centimeter of serum or more, but not effective when the titer was below 2.5. This extract was also highly effective in preventing arteriolonecrotic lesions and death regardless of the antirenin titer. Semipurified renin from hog renal cortex was highly effective in protecting against hypertension and arteriolonecrotic lesions when the antirenin titer was 7.0 AU or above, but ineffective when the titer was less than 7.0. This extract did not protect against death. Semipurified renin from hog whole kidney was inconsistently effective in protecting against hypertension, arteriolonecrosis, and death. The protection against these factors was not well correlated with the antirenin titer produced by semipurified renin from whole hog kidney. Hog crude whole kidney extract did not have an antihypertensive effect. (However,

the antirenin titers were generally below those found necessary for protection in the hog crude renal cortex group.) There was doubtful protection against arteriolonecrotic lesions by hog crude whole kidney extract. Hog crude liver extract had no antihypertensive effect and did not significantly protect against arteriolonecrotic lesions or death.

The antihypertensive effect is directly related to antirenin in the absence of medullary protein. Protection against arteriolonecrotic lesions and death is not directly related to the antirenin titer or the antihypertensive effect. The evidence presented strongly suggests that the nonrenin fraction of the hog crude renal cortex extract, in the absence of medullary protein, is responsible for protection against arteriolonecrotic lesions and death.

22. MAINTENANCE THERAPY WITH VITAMIN B_{12} FOR PATIENTS WITH PERNICIOUS ANEMIA

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Vitamin B_{12} has now been used for more than three years in the treatment of patients with pernicious anemia. Some question has been raised with regard to its ability to maintain these patients in satisfactory remission. Beard and his associates, in a preliminary study of eighteen patients with pernicious anemia maintained with vitamin B_{12}, found the results to be unsatisfactory in seven patients. Mills and Hemsted, after following for one year 144 patients with pernicious anemia maintained on vitamin B_{12}, recommended a dose of 80 μg per month.

We have had the opportunity of following thirty-two patients with pernicious anemia who have been maintained on vitamin B_{12} given intramuscularly for from twelve to twenty-nine months. All patients fulfilled the requirements for the diagnosis of pernicious anemia including a histamine-fast achlorhydria. Fifteen of the group had evidence of subacute combined degeneration of the spinal cord, and eight additional patients had evidence of peripheral neuritis as indicated by paresthesia of the extremities. We have grouped these data regarding the maintenance dose of vitamin B_{12} both as to micrograms per day and as to total dose for the interval of administration. An attempt is made to correlate the patients' clinical, hematologic, and neurologic status with these data.

23. RESULTS OF NITRITE AND THIOSULFATE TREATMENT IN CYANIDE POISONING

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The present treatment is based on results obtained in 227 dogs, an animal more susceptible to cyanides than man. The antidotes used are sodium nitrite and sodium thiosulfate, injected intravenously, one after the other. The function of the former is to produce methemoglobin which will remove cyanide ions from tissues by the formation of cyamethemoglobin. The function of sodium thiosulfate is to convert the remaining cyanide to thiocyanate in the presence of rhodanese. Blood concentration studies show that as methemoglobinemia decreases the amount of thiocyanate increases. The simultaneous injection of the nitrite and the thiosulfate is far more effective than that of either alone. This combination is capable of detoxifying twenty lethal doses of sodium cyanide in dogs. With this therapy, they have survived lethal doses of sodium

cyanide even after respiration has ceased. Results in twenty-nine human cases indicate very definitely that the nitrite-thiosulfate combination is effective in reviving individuals who have been poisoned with hydrocyanic acid, potassium cyanide, or calcium cyanide. Twenty-eight of the twenty-nine victims recovered, and one, moribund, succumbed. The incidence of cyanide poisoning in New York City, San Francisco, Los Angeles, and the United States will be brought up to date.

24. SIMULTANEOUS OCCURRENCE OF PLASMA CELL MULTIPLE MYELOMA AND BOECK'S SARCOID IN A PATIENT WITH PULMONARY TUBERCULOSIS

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The coexistence of plasmacytoma and sarcoidosis in a patient with active pulmonary tuberculosis is extremely interesting because of the possible interrelationship of these diseases. It is suggested that an appropriate stimulus may initiate diverse and simultaneous responses of the reticuloendothelial system.

A 53-year-old white man developed weakness, fatigue, exertional dyspnea, and pallor in 1949. With the findings of a severe anemia, excessive rouleaux formation, myeloid immaturity, many atypical and immature plasma cells in the peripheral blood, hyperglobulinemia, and increased plasma cells in the bone marrow, a diagnosis of plasma cell multiple myeloma was made. The marked bleeding tendency was associated with a severe thrombocytopenia. The spleen and liver were enlarged, and there was a bilateral axillary lymphadenopathy. Biopsy of a lymph node showed the histologic pattern of Boeck's sarcoid. Pulmonary markings were found to be increasing in the roentgenograms. There was no roentgenologic evidence of multiple myeloma. The patient's blood became increasingly difficult to type and cross match so that procurement of blood became extremely difficult. His course was progressively downhill. He developed uncontrollable hemorrhages, pneumonia, and expired ten months after onset of symptoms.

Necropsy showed sarcoidosis of the lungs and lymph nodes; plasma cell myeloma with infiltrations of the liver, spleen, bone marrow, and kidneys, and active tuberculosis with caseation of the lungs. There was evidence of extramedullary hemopoiesis of liver and spleen.

25. STUDIES ON THE TOXICITY OF ANTIBODY-ANTIGEN PRECIPITATES

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Katz (*Science* **91**: 221, 1940) first demonstrated that when the specific antigen is added to blood drawn from sensitized rabbits histamine is released into the plasma. Dragstedt and co-workers (*J. Immunol.* **39**: 537, 1940) observed a similar release following in vitro passive sensitization of rabbits' blood. We have confirmed these findings and demonstrated that the histamine released into the plasma under these circumstances is quantitatively derived from the cells contained in the white cell layer of the centrifuged blood.

As a further extension of these observations we have made a study of the toxicity of antibody-antigen precipitates prepared from rabbits' blood. Rab-

bits were sensitized by repeated injections of crystallized egg albumin or bovine gamma globulin. Serum or plasma was collected from the sensitized animals, and the appropriate antigen added. The antibody-antigen precipitate was collected at the centrifuge and, after washing repeatedly with saline solution, was lyophilized. It was found that when the dried precipitate was suspended in saline solution or dissolved in a strong solution of the antigen and then added to blood from an unsensitized rabbit, histamine was promptly released from the cells of the white cell layer into the plasma in amounts similar to those found when antigen is added to blood from sensitized animals. On the other hand, in control tests little or no histamine was released when the antigen or serum containing the antibody were added to separate samples of blood from unsensitized animals. Release of histamine has not been observed when antigen-antibody precipitates from rabbits' blood are added in vitro to tissues from other animals, although some signs of general toxicity have been seen when the material is given intravenously to intact animals. Thus, further work on this aspect of the problem is needed.

26. METABOLIC EFFECTS IN MAN OF ORALLY AND PARENTERALLY ADMINISTERED COMPOUND F (17-HYDROXYCORTICOSTERONE) AND COMPOUND F ACETATE

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In four separate balance studies on the same normal subject, the comparative metabolic effects of free compound F and compound F acetate, each given both orally and intramuscularly, were determined. In all experiments the dose of each compound was 400 mg. per day. Determinations included daily renal excretion of sodium, potassium, chloride, sulfur, nitrogen, uric acid, glucose, 17-ketosteroids, and formaldehydogenic steroids. On blood or serum, determinations were made for sugar, carbohydrate tolerance, free and esterified cholesterol, total lipid and fatty acid, glutathione, sodium, potassium, chloride, hematocrit, and circulating eosinophils. In a fifth study on the same subject the effects of oral cortisone acetate (400 mg. per day) were determined for comparative purposes.

It was observed that free compound F administered intramuscularly produced intense metabolic changes in all elements of the spectrum studied, while compound F acetate given intramuscularly produced only mild effects. On the other hand, either the free compound or the acetate, when administered orally, resulted in severe metabolic changes comparable to those produced when free compound F was given intramuscularly.

The metabolic effects in man of active compound F include intense renal retention of sodium, chloride and water, potassium diuresis, sharply negative nitrogen and sulfur balances, great loss of carbohydrate tolerance with glycosuria, uricosuria, rapid hemodilution, and disappearance of circulating eosinophils. Urinary excretion of both 17-ketosteroids and formaldehydogenic steroids increases. Acne and "moon face" occurred within two to four days.

The intensity and diversity of the metabolic changes induced by this single steroid are such as to suggest strongly that compound F is the hormone secreted in excess by the normal human adrenal when the latter is stimulated by exogenous ACTH.

27. THE METABOLISM OF FRUCTOSE IN THE LIVER OF NORMAL AND DIABETIC SUBJECTS

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Cori and associates have shown by in vitro studies with liver slices that the phosphorylation of fructose is catalyzed by a separate hexokinase, and that fructose utilization is independent of glucose utilization. Hepatic vein catheterization studies were performed in two diabetic and two normal subjects in order to evaluate the role of the liver in the metabolism of fructose in vivo.

Hepatic venous and femoral arterial blood samples were obtained before, during, and following the intravenous administration of fructose (1 Gm. per kilogram). These were analyzed for fructose, glucose, pyruvic, lactic, citric, and malic acids. Hepatic blood flow was determined by the bromsulfalein method of Bradley. From these data the net splanchnic assimilation of these substances was calculated.

The response to fructose of the diabetic patient deprived of insulin was similar to that of the normal subject. The liver rapidly removed fructose from the blood; approximately half of the administered fructose was taken up by the liver during the period of administration. Pyruvic acid was removed in small quantities during the control period, but during fructose infusion the concentration of pyruvic acid in the hepatic vein was much higher than in the artery. Similar changes were found for lactic acid. Small amounts of citric acid were removed by the liver in the control period, but this pattern of response was not altered by fructose. Malic acid arterial-hepatic vein differences were not significant, and fructose caused no change.

In the two diabetic subjects the elevation of blood glucose following fructose injection did not inhibit the removal of fructose from the blood by the liver, nor was pyruvate formation decreased. These results are consistent with Cori's observation that glucose fails to inhibit the utilization of fructose by liver hexokinase.

These studies indicate that (a) the liver plays an important role in the removal of fructose from the blood stream and converts a significant quantity to pyruvate, presumably by way of the Embden-Meyerhof scheme, and (b) the liver of the diabetic subject, in the absence of insulin, metabolizes fructose in the same manner as does the liver of the normal individual.

28. CONSIDERATION OF THE PLASMA CLEARANCE RATE OF BROMSULFALEIN (C_{BSP}) AS A DYNAMIC INDICATOR OF HUMAN HEPATOCELLULAR FUNCTION

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Investigators need a practical and sensitive method for (a) measuring human hepatocellular function in the resting state, and (b) reflecting acute changes occurring under experimental conditions. The plasma clearance rate of bromsulfalein (C_{bsp}) should meet these requirements, since it represents the product of the hepatic extraction fraction (E_{bsp}) and plasma flow rate (HPF). In obtaining previously reported C_{bsp} values, the observers did not standardize the infusion (loading) rate. In the present experiments intravenous bromsulfalein was infused constantly in every case at 2.5 mg. per minute per square meter; and values for plasma concentration, total removal rate, and clearance were determined from serial blood samples drawn at standard intervals of elapsed time.

Ten male and seven female hospital patients, aged 17 to 68 years, were studied. Twelve "normals" showed basal C_{bsp} values during one hour of infusion, ranging from 503 to 1,055, and averaging 676 ml. per minute. The single patient with conspicuously high C_{bsp} values was a woman of 35 years, with well-controlled diabetes mellitus under insulin therapy. Five "subnormals" had average C_{bsp} values as follows: (1) woman with cerebral palsy and bronchiectasis, early after middle and lower lobectomy of the right lung, 439; (2) woman with bronchial asthma during an acute attack, 430; (3) woman with pernicious anemia in remission, 394; (4) man with portal cirrhosis, 384; and (5) woman with portal cirrhosis, 160.

A striking increase in C_{bsp} to "normal" was noted in the asthmatic patient following spontaneous amelioration of her attack. Conversely, significant decreases below control levels of C_{bsp} have been induced by mild physiologic and pharmacologic stimuli; namely, (1) venous congestion of the lower extremities at 80 mm. Hg, and (2) intravenous injection of 30 μg of epinephrine hydrochloride.

This method is technically satisfactory and relatively easy, lending itself to combination with other physiologic procedures. Initial results appear promising from the standpoints of range of values and sensitivity of responses. A larger series of patients is necessary to establish normal limits for males and females under these standard conditions. Other disease states and pharmacologic agents are to be investigated.

29. CHARACTERISTICS OF DIABETES MELLITUS IN KIMMELSTIEL-WILSON DISEASE

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(INTRODUCED BY RICHARD W. VILTER, M.D.)

In 1936 Kimmelstiel and Wilson described the presence of unique intercapillary lesions in the renal glomeruli of certain diabetic patients. With time their names have become eponymic for the histologic lesion they reported, and clinicians in turn have permuted their finding into a syndrome for long-standing mild diabetes mellitus with coexistent albuminuric kidney disease, hyper-

tension, and edema. However, correlation of the preterminal diagnosis with the autopsy findings has been disappointing.

A study of patients showing the Kimmelstiel-Wilson lesion was made and compared with all other diabetic patients autopsied at the Johns Hopkins Hospital, 1938 to 1950. Of 7,145 patients, 178 had diabetes, and 49 the Kimmelstiel-Wilson Lesion.

The Kimmelstiel-Wilson lesion was not found in the demonstrated absence of diabetes; it appears to be a specific diabetic nephritis. No Kimmelstiel-Wilson patient died in diabetic coma, as compared with 19 per cent among the controls; only two patients had an episode of diabetic acidosis, compared with a 50 per cent incidence of recurrent ketosis among the controls.

The midphase insulin requirement of the Kimmelstiel-Wilson group was fully as great as that of the controls. However, the diabetic course of the Kimmelstiel-Wilson patients was one of remarkable spontaneous amelioration, as contrasted with the controls. Thus the mild degree of diabetes exists only in the latter years of the Kimmelstiel-Wilson patient. There was no difference as to average duration of diabetes between the Kimmelstiel-Wilson group and the controls. This is contrary to the prevalent belief that the Kimmelstiel-Wilson lesion develops in diabetes of long standing.

What Kimmelstiel and Wilson described as a histopathologic entity appears to represent underlying differences in metabolism from those of ordinary diabetes. From its onset the Kimmelstiel-Wilson metabolic disease shows unusual diabetic attributes which include striking rarity of acidosis and progressive amelioration. When these characteristic criteria are added to the usual nonspecific criteria of hypertension, edema, and renal insufficiency, diagnosis should be facilitated, and in such recognition the hazard of hypoglycemia from administered insulin in dosage previously required will be avoided.

30. URINARY EXCRETION OF ADRENOCORTICAL HORMONES IN SURGICAL PATIENTS PROVIDED SUPPLEMENTS OF ASCORBIC ACID

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Earlier investigations have indicated that ascorbic acid may be involved in the production and secretion of steroid hormones by the adrenal gland. This interrelationship has been studied in two groups of human subjects undergoing the stress of major surgical operations. Each group included seven patients selected so that individuals with similar types of illness and of comparable duration and severity were equally distributed between the two groups. The first group received a daily supplement of 1.0 Gm. ascorbic acid administered intravenously daily throughout preoperative and postoperative periods of investigation. The second, a control group, received no supplementary ascorbic acid. The urinary excretion of formaldehydogenic steroids and the blood eosinophile count were determined daily. In addition, determinations were made of the plasma concentration of ascorbic acid and the rate of urinary excretion of the vitamin.

In all patients of both groups the stress of the surgical procedure was associated with a decrease in circulating eosinophiles to zero or to very low counts. There was a three- to fivefold increase in the rate of excretion of formaldehydogenic steroids and almost total disappearance of ascorbic acid from the urine on the day of operation. In the group of control subjects the urinary excretion of the steroid hormones decreased to preoperative levels

within the first two to three postoperative days. Administration of ascorbic acid was associated with increased excretion of steroids, sustained throughout the first five postoperative days, and significantly greater than in control subjects from the second through the fifth day. A decrease in plasma ascorbic acid occurred almost uniformly in the control group coincident with the surgical procedure, but did not occur in patients who received the vitamin supplement. Only 10 to 30 per cent of ascorbic acid administered on the day of operation was recovered in the urine. This contrasted with excretion of from 50 to 90 per cent in the late postoperative period.

The significance of these findings is discussed.

31. ETIOLOGY OF PRE-ECLAMPSIA-ECLAMPSIA

4-Sodium Chloride Test for the Diagnosis of Pre-eclampsia

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Studies of sodium, potassium, and chloride metabolism have been made in patients with toxemia of pregnancy. The object has been to determine if any of these ions is toxic to the pregnant patient, and also to determine if one could be used as a test to determine whether or not the patient has pre-eclampsia. A retention catheter is inserted, and 1,000 ml. of a 2.5 per cent sodium chloride solution are given intravenously in thirty to forty minutes. The bladder is emptied fifteen minutes after the completion of the injection and the urine is discarded. Two one-hour collections of urine are obtained. It was found that the concentration of sodium in the urine after the intravenous injection of sodium chloride gave the best differentiation between pre-eclampsia and hypertensive disease in pregnancy. The values are shown in Table I.

Table I

	URINARY SODIUM CONCENTRATION RANGE		URINARY CHLORIDE CONCENTRATION RANGE	
DIAGNOSIS	MG. %	MEQ. PER LITER	MG. %	MEQ. PER LITER
Pre-eclampsia	-332	-144	-518	-146
Essential hypertension	332-506	144-220	518-768	146-217
Normal	over 506	over 220	over 768	over 217

It was also noted that if the patient was on a normal chloride intake the determination of the chlorides gave almost as good results as the sodium determination.

There have been no ill effects noted from a single injection of this amount of sodium chloride. If the patient had evidence of pulmonary edema, the injection was not made. A few patients have had only 500 ml. of a 2.5 per cent chloride solution injected intravenously. As more data are accumulated, this amount may be found to be sufficient.

32. THE DISCRIMINATION OF CARDIAC FORCES WITH THE TORSION BALLISTOCARDIOGRAPH

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(Introduced by Dr. William Menninger)

The paper will consist of a description of the torsion ballistocardiograph, its use in 150 subjects, and the formulas which we have developed for discriminating opposing forces, occurring simultaneously, in the head-foot and lateral plane.

By careful placing of the ascending and descending aorta, under fluoroscopic control, in relation to the center of torque it is feasible to analyze the component parts of the ballistocardiogram in a manner not possible with other instruments. The movement of blood in the ascending aorta creates a vector which is obscured by the vector formed by movement of blood down the descending aorta. Cases will be presented in which the torsion ballistocardiogram (TBCG) has separated these forces.

The TBCG has been recorded in twelve hypertensives while hypertensive and when in etamon shock. These will be presented, also.

33. THE DIAGNOSIS OF AMEBIASIS, THE ROLE OF THE COMPLEMENT FIXATION TEST, AND THE INCIDENCE OF THE DISEASE IN THE CHICAGO AREA

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Since 1946, 2,836 patients have been studied for the presence of amebiasis. This group represents a selected population sample referred to the laboratory because of the presence of active gastrointestinal tract symptoms or fever of undetermined origin. Rigid criteria of identification were employed requiring the positive identification of characteristic cysts, or the identification of trophozoites as *Endamoeba histolytica,* only if by special treatment of the stools, encystment was produced. 7.33 per cent of the patients studied were found to be infected. A correlation of the frequency of identification with the number of stool specimens studied per patient showed that in 55 per cent of instances the diagnosis was made on the first specimen examined, and 97 per cent by the time three specimens were examined.

Complement fixation tests were performed by the National Institute of Health on 458 of the patients studied. Sixty of these patients had positive stools. Nineteen patients had both positive stools and positive complement fixation tests; forty-one patients had positive stools and negative complement fixation tests; one hundred six patients had positive complement fixation tests and negative stools. It is concluded that there is no specific correlation between the complement fixation test and the stool examination which gives constructive value to the component fixation test as a diagnostic procedure.

34. EVIDENCE OF DIRECT ACTION OF ADRENOCORTICAL STEROIDS ON CONNECTIVE TISSUE: THE EFFECTS OF SYSTEMIC ADMINISTRATION OF ACTH AND CORTISONE, AND OF INTRA-ARTICULAR CORTISONE AND COMPOUND F ON THE JOINT FLUID IN RHEUMATOID ARTHRITIS

IVAN F. DUFF, M.D., WILLIAM D. ROBINSON, M.D., AND ELIZABETH M. SMITH, B.S. (BY INVITATION), ANN ARBOR, MICH.

The joint fluid in untreated rheumatoid arthritis is characterized by increased white cells, predominately polymorphonuclears, and deterioration of mucin, manifested by poor mucin-clot formation on addition of dilute acetic acid, and a lowered viscosity, despite a relatively normal muco-polysaccharide concentration determined turbidometrically. During systemic administration of ACTH and cortisone, there is prompt decrease in total white cells, due entirely to a decrease in polymorphonuclears, and evidence of improvement in mucin quality.

Repeated daily intra-articular injections of 10 to 25 mg. of cortisone acetate produces a similar change in joint fluid cytology and improvement in quality of mucin and viscosity. These changes occur chiefly in the treated joint, and are not seen to the same degree in affected contralateral joints. Similar changes frequently but not uniformly occur after a single intra-articular injection of 50 mg. of cortisone acetate. Twenty-five and 50 mg. of compound F acetate produces more prompt and better sustained decreases in polymorphonuclear cells, and apparently greater and more sustained increases in viscosity, as compared with cortisone acetate.

Joint fluid viscosity is dependent primarily on the concentration and physical characteristics of hyaluronic acid. Increases in viscosity can be attributed to increase in concentration or increase in degree of polymerization of this muco-polysaccharide. By serial dilutions, we have confirmed Ragan and Meyer's (*J. Clin. Investigation* **28**: 56, 1949) observation that the logarithm of the viscosity bears a linear relationship to polysaccharide concentration, so that the ratio $\frac{\text{Log Viscosity}}{\text{Concentration}}$ is an index of the degree of polymerization of the hyaluronic acid. This ratio increases following intra-articular injections of compound F acetate in doses of 25 and 50 mg. and of cortisone acetate in doses of 50 milligrams.

These results support the concept that adrenocortical steroids exert their favorable action in rheumatoid arthritis by a direct effect at the connective tissue level, and in addition to suppressing inflammatory changes, influence the altered chemistry of connective tissue toward a more normal pattern.

35. CALIBRATED ELECTROSTETHOGRAPHY FOR THE LOCALIZATION OF HEART SOUNDS AND MURMURS OVER THE PRECORDIUM

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A four-beam cathode ray tube assembly permits the photographic recording of three areas of the precordium simultaneously together with the electrocardiogram. The calibration technique consists of a specially designed piston phone which provides an adjustable source of power in dynes, with a known waveform, and a frequency range of 20 to 600 cycles per second. This power,

which is calculated from absolute units, is used to standardize a secondary calibrator by which the microphones, amplifiers, and cathode ray tubes can be quickly adjusted to any desired sensitivity in dynes per centimeter deflection on the photographic record. The recording speed is 20 cm. per second, and the traces are of sufficient sharpness to permit study of frequencies up to 1,000 cycles per second, or time differences of a few thousandths of a second. A study of the timing and frequency characteristics permits an accurate analysis of heart sounds and murmur localization. For study, the records are arranged on a three times enlargement of the roentgenographic outline of the heart, and usually twenty-seven to thirty different locations are recorded.

36. DETERMINATION OF THE BLOOD FLOW THROUGH NON-VENTILATED PORTIONS OF THE NORMAL AND DISEASED LUNG

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The respiratory function of the lungs depends on adequate alveolar ventilation and the perfusion of alveoli with venous blood. Alveolar ventilation may be prevented by atelectasis, or alveoli may be replaced by fibrous tissue or neoplasm. The oxygen content of the blood returning to the pulmonary veins from this diseased tissue will be the same as that of pulmonary artery blood. In a physiologic sense, the circulation through the nonventilated lung represents an arteriovenous shunt.

The purpose of this study was to determine the magnitude of the shunt in various types of pulmonary disease. Oxygen tensions of alveolar air and arterial blood were measured after breathing 100 per cent oxygen for twenty minutes. The latter was determined with the polarograph. The method requires the use of high oxygen tension in order to saturate completely the hemoglobin with oxygen and to obviate the effect of unequal alveolar ventilation and membrane gradient. If no shunt exists the tension of oxygen in the alveoli and arterial blood should be the same. The magnitude of the shunt can be calculated from the difference in oxygen tension between the alveolar air and arterial blood and the arteriovenous oxygen difference.

In eleven normal men the mean difference in oxygen tension between the alveolar air and arterial blood was 17 mm. Hg, a value which represents a shunt of 1 per cent of the total pulmonary blood flow. In sixteen patients with collapse therapy for tuberculosis that resulted in reduced oxygen uptake of the involved lung as demonstrated by bronchospirometry, the shunt was 4.6 per cent. The following values were obtained for other groups of patients: eleven cases of advanced bilateral tuberculosis, 3.6 per cent; twelve cases of bronchogenic carcinoma, 7.1 per cent; eight cases of convalescent pneumonia, 7.5 per cent; six cases of pleural effusion, 8.3 per cent; thirteen cases of pulmonary emphysema, 5.1 per cent; ten cases of hepatic cirrhosis, 5.8 per cent. The striking feature of these results is the finding that in extensive chronic pulmonary disease with obvious destruction of function in large areas of lung the pulmonary shunt can be extremely small.

37. THE GENESIS OF CARDIOVASCULAR CHANGES ASSOCIATED WITH THE VALSALVA MANEUVER IN NORMAL PERSONS

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(Introduced by L. N. Katz, M.D.)

The Valsalva maneuver in normal persons is characterized during the period of sustained straining by a diminished systemic pulse pressure and an associated tachycardia. After cessation of straining, the systemic blood pressure falls briefly, and then a pronounced overshoot above control values follows, associated with a reflex bradycardia. The venous pressure during the straining period increases progressively because blood is prevented from entering the heart as a result of the increased intrathoracic pressure. When straining is discontinued the venous pressure falls rapidly. There is, at the same time, an increased inflow of the blood previously held back. The precise mechanisms of these observed changes were investigated.

The Valsalva maneuver was performed in several normal persons before and after the adminstration of tetraethyl-ammonium chloride (TEAC). Following TEAC, the response *during* straining was not altered significantly. However, *after* the straining period, the overshoot in pressure was absent and the heart rate increased. The Valsalva maneuver after atropine, in another group of normal persons, gave essentially the same response as before atropine was given, except that after straining the pulse accelerated instead of slowing, despite the presence of the usual blood pressure overshoot.

From these results, it is concluded that (a) the reflex bradycardia accompanying the blood pressure overshoot in normal persons is mediated by efferent cholinergic fibers in the vagus going to the heart, since it was blocked by atropine; (b) reflexogenic vasoconstriction must be important in the causation of the blood pressure overshoot since, in the normal person, the expected overshoot was absent after TEAC. The tachycardia seen after the maneuver, when either atropine or TEAC is given, may also be reflexogenic as a response to the venous engorgement. When the vagus efferent pathways are operative and a blood pressure rise occurs after straining, the arterial pressor receptors reflexly produce a bradycardia which overbalances the tachycardia which would otherwise result from the venous engorgement.

38. ATHEROSCLEROSIS

AN IN VITRO DEMONSTRATION OF PHENOMENA RESEMBLING THE EARLY LESIONS OF ATHEROSCLEROSIS

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Many physical and chemical variations have been statistically correlated with atherosclerosis in case series. No one varient, however, can be identified as consistently present in all cases with atherosclerosis and absent in all non-atherosclerotics.

An effort was made to produce in vitro changes in vessel walls resembling early atherosclerosis by combining and controlling those factors statistically associated with atherosclerosis.

Apparatus was developed for pulsating oxylated blood against normal fresh strips of human aorta obtained at autopsy.

Twenty-two blood samples from normal young adults and twenty-four similar samples from myocardial infarction cases or diabetics with vascular disease were obtained. These were pulsated against strips of normal aorta for seventy-two hours at 300 to 0 pressure, and 80 per minute pulse rate. Careful relative asepsis and physiologic temperature was maintained throughout.

At the end of each experiment, the aortic strips were removed, fixed, and frozen sections were cut. These sections were stained for fat and examined under normal and polarized light.

The greatest difference between bloods from control and atherosclerotic sources was manifest in unstained sections under polarized light microscopy. Seventy-five per cent of the aortic strips pulsated with blood of disease origin revealed marked depositions of birefringent particulate material in the vessel wall, whereas those strips pulsated with control bloods revealed no change in 95.5 per cent and very minimal deposition in the rest. The aortic strips in the "positive" group closely resembled early atheromas similarly prepared. A statistical analysis has revealed that this difference observed in the two groups would occur by chance less than one time in a thousand.

Conclusions.—(a) Deposits of birefringent material are produced in vitro in a vessel wall by pulsating the vessel with the proper blood sample. (b) The deposits so produced resemble early atheromas. (c) The deposits are absent or minimal when the blood is obtained from normal young adults, and present in most cases when the blood is obtained from cases with myocardial infarction or vascular disease. (d) Analysis of these data has revealed statistical significance.

39. CATABOLISM OF ADRENAL CORTICAL STEROIDS AFTER INTRAMUSCULAR AND ORAL ADMINISTRATION

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A comparison of the effect of intramuscular and of oral administration of compounds B, E acetate, F and F acetate, and S acetate (Reichstein), on the urinary excretion of 17-ketosteroids and formaldehydogenic steroids has been made. Each of these steroids was administered to normal subjects at a dosage level of 200 to 400 mg. per day.

Compounds B and E acetate caused a small decrease from base-line levels in excretion of 17-ketosteroids when given intramuscularly and a small increase when given orally. On the other hand, excretion of formaldehydogenic steroids increased when each compound was administered by either route.

Free compound F administered intramuscularly caused an initial decrease followed by an increase in excretion of 17-ketosteroids. When given orally, free compound F caused a considerably greater increase in excretion of 17-ketosteroids. In contrast, excretion of formaldehydogenic steroids was greater during intramuscular administration of the compound.

Compound F acetate caused a marked decrease in excretion of 17-ketosteroids when given intramuscularly and a marked increase when given orally. Excretion of formaldehydogenic steroids was much greater during oral administration of the compound.

The greatest increase in excretion of 17-ketosteroids was observed during oral administration of compound S acetate, but there was no significant change during intramuscular administration. Oral administration of compound S acetate, at the same dosage level, to a patient with Addison's disease caused a

comparable rise in excretion of 17-ketosteroids. A patient with atrophic cirrhosis of the liver, given compound S acetate orally, showed a very much smaller increase in excretion of 17-ketosteroids.

Since upon oral administration the steroids pass through the liver in high concentration, these observations suggest that the liver plays a major role in facilitating conversion of adrenal steroids to 17-ketosteroids.

40. MAGNESIUM, POTASSIUM, PHOSPHORUS, CHLORIDE, AND VITAMIN DEFICIENCY AS A RESULT OF PROLONGED USE OF PARENTERAL FLUIDS

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The case of a 33-year-old white woman with mutliple electrolyte and vitamin deficiencies is being presented. She began to vomit five days before the delivery of her eighth child and continued to vomit post partum. A diaphragmatic hernia was repaired seven days after delivery. She continued to vomit and was given parenteral fluids for several months. Three months after the operation she was admitted to the University of Minnesota Hospital. She was cachectic, dehydrated, semiconscious, and appeared moribund. Her tendon reflexes were absent, the Babinski sign was positive on the left, and there was marked weakness of all muscles.

Hypochloremic, hypokalemic alkalosis was found on admission (serum chloride 60 meq. per liter, potassium 1.9 meq. per liter, sodium 117 meq. per liter, and carbon dioxide 41 mM per liter). The hypokalemic alkalosis was corrected. The serum sodium returned to normal as a result of shift of sodium from cells to serum. At this time hypophosphatemia (inorganic phosphorus 0.6 mg. per 100 ml.) was recognized and corrected. As the alkalosis and hypophosphatemia were improving, she developed fibrillary twitchings, gross muscle tremors, and choreiform movements of the extremities, jaw, tongue, and facial muscles. She was unable to talk or eat. At this time a magnesium deficiency was recognized (serum magnesium 1.19 meq. per liter). Magnesium sulfate was administered intramuscularly, and the choreiform movements and twitchings diminished dramatically and finally ceased. She was able to speak clearly and began eating. The serum magnesium level returned to normal. Ten days later severe hyperesthesia and burning of the lower extremities began. She had received vitamin B complex daily up to this time. However, after the administration of large doses of calcium pantothenate orally, the hyperesthesia and burning disappeared overnight. In a few days she was able to walk and was discharged from the hospital.

This patient presented with remarkably severe electrolyte disturbances. The magnesium deficiency and the response to therapy are particularly noteworthy. The prompt relief of hyperesthesia afforded by calcium pantothenate is also striking. Prolonged parenteral fluid administration is hazardous unless multiple ions are administered to prevent deficiency.

41. DEPRESSOR EFFECT OF CHRONIC SALT DEPLETION IN RENAL HYPERTENSIVE DOGS

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The role of sodium in the pathogenesis of hypertension remains a major unsolved problem. Its importance is emphasized by recent demonstrations that low sodium diets are therapeutically efficacious in many cases of human hypertension. Attempts to elucidate the mechanism of these depressor responses by experimental studies in renal hypertensive dogs have reached an impasse, since no blood pressure fall has been elicited in such animals fed low sodium diets. In view of these disparate findings, we undertook to study this problem further in Goldblatt dogs.

Five different types of experiments were accomplished, with the following results: (a) In accord with previous findings, treatment of established benign Goldblatt hypertension with a low sodium diet (Mead Johnson's Lonalac) failed to effect a reduction of blood pressure, even when continued for as long as nine months. (b) A combination of low sodium diet plus chronic salt depletion (via peritoneal lavage) consistently resulted in a lowering of blood pressure from hypertensive to normotensive levels. This depressor response was associated with a fall in plasma sodium concentration to hyponatremic levels, and a rise in hematocrit. (c) Unlike the foregoing animals with established renal hypertension, control normotensive dogs exhibited no blood pressure alteration in response to combined low sodium diet plus salt depletion. (d) A prolonged period of subsistence on an exclusive low sodium diet did not prevent the development of the malignant hypertensive syndrome following complete ligation of both renal arteries. (e) Studies in several animals yielded inconsistent data with reference to the possible prophylactic effect of low sodium diets against benign Goldblatt hypertension produced by partial ligation of both renal arteries.

These experiments demonstrate that sodium withdrawal, complete enough to effect a significant sodium depletion, elicits a depressor response in renal hypertensive dogs, as in hypertensive man. This finding, together with the different responses of normotensive and hypertensive animals to sodium depletion, reinforces the concept that sodium plays a significant role in the pathogenesis of hypertension.

42. RIGHT VENTRICULAR HYPERTROPHY: A COMPARISON BETWEEN RIGHT VENTRICULAR PRESSURE AND THE TWELVE LEAD ELECTROCARDIOGRAM IN TWENTY-SIX PATIENTS

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(Introduced by Warren B. Cooksey, M.D., and Hugo A. Freund, M.D.)

The diagnosis of right ventricular hypertrophy (RVH) can be made in the presence of typical findings on the electrocardiogram. However, it has been shown that the absence of the typical findings in the augmented unipolar and precordial leads does not exclude the diagnosis of right ventricular hypertrophy. With this in mind, it was thought that a study of the right ventricular pressure might reveal hypertrophy when it could not be read from the electrocardiogram. The technique of Cournand for venous cardiac catheteriza-

tion was employed, and the pressure apparatus used was an electromanometer. The latter is standardized against a mercury manometer. Twelve lead electrocardiograms (3 standard, AVR, AVL, AVF, V-1-6) were taken on all patients, and in a few, V-3 on the right. The twenty-six patients studied included congenital heart anomalies, rheumatic heart disease, and two cases of polycythemia. Seventeen presented electrocardiograms which were diagnosable as right ventricular hypertrophy (fifteen diagnosed from V-1 and 2 alone, or V-1 and 2 plus V-5 and 6, AVR, or Standard lead changes; one from AVR and V-5 and 6, without V-1 and 2 findings; one was suggestive of RVH from V-5 and 6 alone). All seventeen had an elevated right ventricular pressure.

Nine patients had no evidence of RVH on their electrocardiograms. Three of these had an elevated right ventricular pressure (one had a patent ductus arteriosus and an interventricular septal defect; the second had a single ventricle and probably should not be considered solely as right ventricle; the third had a polycythemia and a hypertension which revealed left ventricular hypertrophy on the electrocardiogram).

There seems to be a definite correlation between the elevated right ventricular pressure and the electrocardiographic changes of RVH. In one case an elevated right ventricular pressure occurred where the electrocardiogram showed no evidence of RVH. More cases are to be studied with this point in mind.

43. THE EFFECT OF SWEATING ON NORMO- AND HYPERTENSIVE SUBJECTS

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In order to determine the effect of loss of body water, with relatively little loss of serum sodium and chloride, on the blood pressure in normotensive and hypertensive subjects, eight of the former and seven of the latter were subjected to a sweating procedure. For this purpose, a hot room was used, the temperature being set at 115° to 118° F. The subjects remained in the hot environment until they lost about 4 per cent of their control body weights. Determinations of the blood pressure, serum sodium, potassium, and chloride, and body fluid spaces were carried out before and after the sweating procedure. For the latter, the T-1824, thiocyanate, and antipyrine techniques were used.

The blood pressure declined slightly in both groups of subjects while they were in the hot room, but returned to control levels within an hour after termination of the sweating procedure. The normotensives lost an average of 3.95 kg. of body weight, and the hypertensives 2.57 kilograms. Most or all of the weight loss was due to loss of body water; the mean decreases in antipyrine space were 3.48 and 2.78 liters, respectively. Most of the fluid lost apparently came from the extracellular fluid space (thiocyanate space), there being relatively little change in the blood volume. The diminution in thiocyanate space was comparable to that observed by us in eight hypertensive patients being treated with the rice-fruit diet; in four of the latter, there was a dramatic decline in blood pressure.

In the hypertensive subjects, the loss of serum sodium and chloride per liter of extracellular fluid lost was about twice as large in the subjects receiving the rice-fruit diet as in the subjects who were sweated. In addition, there is some indication that hypertensive subjects conserve sodium and chloride, when subjected to the sweating procedure, somewhat better than do normotensive subjects.

We conclude that the depressor effect of salt-depletion regimens is probably not due solely to loss of body water and that a specific electrolyte effect is, in part at least, responsible.

44. THE EVALUATION OF USE OF REGITINE (C7337) AS A NEW PHARMACOLOGIC TEST FOR PHEOCHROMOCYTOMA

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For patients with pheochromocytoma and paroxysmal hypertension, histamine, Mecholyl, and tetraethyl-ammonium chloride have been used to induce attacks similar to spontaneous attacks. In cases of pheochromocytoma and persistent hypertension, however, attacks are negligible or absent. In such cases piperoxan (Benodaine), ergot alkaloids, and dibenamine have indicated a pheochromocytoma by lowering blood pressure when the pressor substance is circulating in the blood. However, these drugs are not infallible and are hazardous to give. Grimson and associates suggested that Regitine (C7337) may be less hazardous.

Four hundred fifteen pharmacologic tests were performed in our laboratory on 204 patients suspected of having pheochromocytomas. In 212 tests 5 mg. of Regitine was given either intramuscularly or intravenously. Results were compared with results of 203 tests with piperoxan or histamine on the same patients.

In twenty patients with normal ocular fundi Regitine had a variable but negligible effect on blood pressure. In the 179 patients with hypertension, grade I to IV (Wagener-Keith classification) Regitine produced no change, a slight rise, or in the majority of patients a slight reduction in the blood pressure. However, in one patient with uremia and in one who was heavily sedated, the depressor effect of Regitine was sufficient to indicate incorrectly the presence of a pheochromocytoma.

Five patients had proved pheochromocytomas. Two of these had paroxysmal hypertension, and Regitine had no effect on blood pressure between attacks. The other three had persistent hypertension. The intramuscular administration of Regitine gave a positive result in one, an equivocal result in another, and a negative result in the third. In contrast, the intravenous administration of Regitine to the last two patients produced a sharp prompt fall in blood pressure similar to that produced by piperoxan. Because of this the intravenous administration of Regitine should be the method of choice.

Avoidance of sedation before any of these pharmacologic tests is most important.

Regitine proved easy to administer and produced few if any subjective symptoms. The pressor effect of Regitine was considerably less than that of piperoxan, and therefore Regitine is safer. It seems to be another valuable testing substance for pheochromocytoma.

45. FURTHER STUDIES ON THE CARDIAC OUTPUT IN ACUTE MYOCARDIAL INFARCTION

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(INTRODUCED BY GEORGE K. FENN, M.D.)

While clinical and experimental evidence would indicate that the cardiac output is low in cases of recent myocardial infarction both with and without shock, precise information on this point is meager. The possibility of peripheral vasodilatation has been mentioned by some as a contributing factor in the production of low blood pressure in occasional patients with acute myocardial infarction and shock.

Nine cases of clinically severe myocardial infarction have been studied within the first twenty-four to forty-eight hours. The cardiac output was measured at the bedside by the dye dilution technique so that only one arterial and one venous puncture were necessary. No harmful effects were apparent. Arterial pressures were recorded with a strain gauge manometer. The venous pressure, circulation time, plasma volume, and hematocrit were also determined.

In general, the cardiac index was low, particularly in those patients who did not survive the first week. The total peripheral resistance was elevated in all cases except one, in which it was 870 dynes cm.$^{-5}$ sec. This patient had just recovered from a period of profound hypotension. In one patient with a blood pressure of 65/43, a cardiac output of 1.22 liters per minute and a cardiac index of 0.794 liters per minute per square meter the TPR was still elevated to 3540 dynes cm.$^{-5}$ sec. The venous pressure in this patient was 60 mm. of saline but rose during pressure on the abdomen.

There was some tendency to a reduction of the plasma volume as expressed in milliliters per kilogram. This seemed to correlate with increasing values for the venous pressure, but did not apparently correlate with an increased hematocrit.

It may be tentatively concluded that in acute myocardial infarction the cardiac output is generally reduced and the TPR increased. This situation obtained in one patient who was in shock. The findings in another patient, however, would suggest that there may occasionally be a decrease in the total peripheral resistance. Further data are necessary and the study is still in progress.

46. INTRACTABLE CONGESTIVE HEART FAILURE DUE TO MYOCARDITIS: A CLINICAL AND PATHOLOGIC ENTITY

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A clinical syndrome characterized by prolonged, intractable heart failure, occurring in the absence of signs of valvular disease, hypertension, pericarditis, or coronary arteriosclerosis has been observed frequently during the past five years in the Barnes Hospital. The majority of the patients have been men between the ages of 30 to 60 years. The clinical features, in addition to the signs of congestive failure, have included cardiac hypertrophy, low-grade fever, bundle branch block, and low pulse pressure. Signs of pulmonary or peripheral embolization have not been uncommon.

The principal post-mortem lesions have been cardiac enlargement, often of marked degree (700 to 800 Gm.), myocardial fibrosis, mural thrombi, and pulmonary and systemic infarcts. In two cases of relatively short duration, isolated myocarditis was present; in one of these cardiac enlargement and myocardial fibrosis were marked, suggesting recurrent myocarditis.

Cases similar to these have been reported by Levy, Dock, Smith and Furth, Davies, and others. Although alcoholism and/or nutritional deficiency have been advanced as having possible etiologic significance, available evidence does not support either of these factors in the causation of the syndrome. The finding of isolated myocarditis in the two early cases already alluded to suggests that a progressive or recurrent noxious stimulus, such as infection or hypersensitiveness, may play a role in the development of the disease.

Experience with the first few cases of this syndrome has led to increased awareness and to correct ante-mortem diagnoses. Because of the increase in the occurrence of cases conforming to the criteria of the syndrome—an increase substantiated by an extensive analysis of all of the post-mortem examinations performed in this institution over a long period of years—it is believed that attention should be called to it again. It is hoped that continued observation and study may yield definitive knowledge of the etiology of the pathologic changes.

47. THE EFFECT OF PAS ON THE DEVELOPMENT OF RESISTANCE OF TUBERCLE BACILLI TO STREPTOMYCIN

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This investigation was a study of the effect of para-aminosalicylic acid (PAS) upon the development of resistance of tubercle bacilli during streptomycin or Dihydrostreptomycin therapy in far-advanced pulmonary tuberculosis. A total of seventy-four patients were studied in three categories: (1) Dihydrostreptomycin 1.0 Gm. daily, (2) Dihydrostreptomycin 1.0 Gm. and PAS 10.0 Gm. daily, and (3) streptomycin 1.0 Gm. and PAS 10.0 Gm. daily. Treatment extended over a ninety-one day period, and observation continued for three months thereafter. Sensitivity tests were performed on cultures taken at ten specified intervals during the entire course of study.

Results are shown graphically for each category. Prior to therapy, cultures in all categories were sensitive. The most striking change occurred in the Dihydrostreptomycin group during the first eight weeks of drug administration and during the first ten weeks in the other two groups receiving PAS. Thereafter there was relatively little change in any of the groups.

The Dihydrostreptomycin group showed by far the greatest proportion of resistant cultures, 44 per cent at the end of twelve weeks and 45 per cent after twenty-seven weeks. The Dihydrostreptomycin and PAS group showed only 4 per cent resistant at the end of twelve weeks, and 6 per cent after twenty-seven weeks. The streptomycin and PAS group showed 12 per cent resistant after twelve weeks and 26 per cent at the end of twenty-seven weeks.

At the end of twelve weeks the Dihydrostreptomycin group showed 14 per cent sensitive cultures compared to 42 per cent in each of the other two groups. After twenty-seven weeks, 22 per cent of the Dihydrostreptomycin group were sensitive as compared to 44 per cent in the Dihydrostreptomycin PAS group, and 16 per cent in the streptomycin PAS group. Also at this time the Dihydrostreptomycin group show 28 per cent negative cultures, the Dihydrostreptomycin and PAS 44 per cent, and streptomycin and PAS 53 per cent.

These results indicate that the addition of PAS delays or inhibits the development of resistance of the tubercle bacillus to streptomycin.

48. INHIBITION BY NITROGEN MUSTARD OF THE GENERALIZED SHWARTZMAN REACTION AND OF BILATERAL RENAL CORTICAL NECROSIS PRODUCED WITH S. MARCESCENS TOXIN IN CORTISONE-TREATED RABBITS

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The generalized Shwartzman reaction, characterized by bilateral hemorrhagic renal necrosis, occurs in 75 to 100 per cent of rabbits following two intravenous injections of Shear's *S. marcescens* toxin spaced twenty-four hours apart. Since it is known that nitrogen mustard (HN_2) inhibits the local Shwartzman reaction in the skin, a study of the effect of HN_2 on the generalized Shwartzman reaction has been carried out.

Nitrogen mustard in dosages of 1.5 to 1.75 mg. per kilogram completely prevents the generalized Shwartzman reaction in all rabbits when given seventy-two hours prior to the initial injection of endotoxin. When HN_2 was given within twenty-four hours of the first injection of endotoxin, no protective effect could be demonstrated. The inhibitory effect of HN_2 was abolished when the femoral bone marrow was protected from the effect of HN_2 by a clamp on the lower abdominal aorta at the time of injection. Hematologic studies suggest that here, as in the local Shwartzman reaction, the protective effect of HN_2 is brought about by suppression of the bone marrow, with elimination of circulating polymorphonuclear leucocytes.

Following the demonstration in this laboratory that bilateral renal cortical necrosis can be produced by a single injection of bacterial endotoxin in cortisone-treated rabbits, a study of the effect of HN_2 on this phenomenon was undertaken. It was found that HN_2 regularly inhibits the development of renal necrosis when given in dosages similar to those required for inhibition of the Shwartzman phenomenon. In this case the protective effect of HN_2 was partly but not completely abolished by clamping the abdominal aorta at the time of administration of HN_2. The findings suggest that the pathogenesis of bilateral renal necrosis in cortisone-treated rabbits given a single injection of bacterial endotoxin may be similar to that of the generalized Shwartzman reaction.

49. THE EFFECTS OF CARINAMIDE ON PARA-AMINOSALICYLIC ACID BLOOD LEVELS

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Two conflicting reports have appeared from England within the past year concerning the use of carinamide for elevating para-aminosalicylic acid (PAS) blood levels. It was the purpose of this study to determine the effects of such therapy on fourteen cases of far-advanced pulmonary tuberculosis. A consistently high PAS level is necessary to prevent the development of acid-fast bacilli which are resistant to streptomycin.

The patients were given fourteen grams of PAS daily in three equally divided doses with meals. Varying dosages of carinamide up to nine grams daily were used. Keane's colorimetric method was used to determine the PAS levels at hourly intervals. Carinamide in the above dosage to controls gave a false positive PAS reaction of 0.5 mg. per cent. With PAS alone, the blood levels rose to 10 to 12 mg. per cent within an hour and returned to 2 to 4 mg. per cent at the end of the four-hour period. With carinamide, the levels of PAS usually rose to 14 to 15 mg. per cent at the end of one hour and did not fall below 6 to 9 mg. per cent at four hours.

The elevation of temperatures fell and chest roentgenograms showed improvement. The patients, however, did not feel better, and several developed acidosis. All had gastritis with nausea, vomiting, and diarrhea at various times. No antacids were used. Tincture of belladonna gave slight relief. All patients developed hypoprothrombinemia (40-50 per cent of normal) which was corrected with vitamin K orally. The urine of four patients had albumin, a positive qualitative Benedict's, and a positive Bial reaction, interpreted to be due to pentose. These four patients had normal glucose tolerance tests.

It is concluded that the blood levels of PAS may be significantly elevated by the concurrent use of carinamide. The increased incidence of toxic reactions such as nausea, vomiting, diarrhea, and acidosis makes the routine use of carinamide with PAS and streptomycin unwise.

50. PSYCHOGENIC DYSPNEA IN CARDIOVASCULAR DISEASE

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A 66-year-old man with hypertensive cardiovascular disease was studied because of unusual dyspnea of five months' duration which had not responded to the usual measures designed to correct cardiac decompensation.

Examination revealed a type of respiration best described as "breathing like a fish" without any orthopnea, pulmonary congestion, peripheral edema, or other evidence of congestive failure. The heart was generally enlarged, especially in the region of the left ventricle, and the aorta was elongated and tortuous. The blood pressure was repeatedly elevated in the range of 165/105. Moderate cyanosis of the lips was present.

Several determinations of the venous pressure were within normal limits. The circulation times were always prolonged. The blood counts were within normal limits and the arterial oxygen saturation was over 95 per cent on two occasions. Renal function was adequate, and the electrocardiogram showed evidence of left ventricular enlargement.

There was no change in circulation time, venous pressure, symptoms, or physical findings after complete digitalization, salt restriction, bed rest, and the administration of mercurial diuretics.

Measurements of the total lung volume and its subdivisions revealed values within the normal range except for a slight increase in the residual/total ratio. Effective alveolar ventilation was about 17 liters per minute due chiefly to the large tidal volume of over 1150 c.c. with a respiratory rate of 16. As a result of this increased ventilation the pCO_2 fell to 25 mm. Hg, and the CO_2 combining power fell to 23.4 meq. per liter, while the pH rose to 7.58. Despite these changes, the only associated electrolyte disturbance was an increase in the chloride to 116 meq. per liter. The serum sodium, potassium, calcium, and protein determinations were not abnormal.

With reassurance and partial hypnosis, the hyperventilation was abolished, and the above abnormalities tended to revert toward normal.

Further interviews established the fact that the patient was suffering from a severe "cardiac neurosis." The onset of the dyspnea was associated with sexual intercourse on an extramarital basis. He was reassured and instructed in measures to forestall or modify the hyperventilation. Two weeks following discharge from the hospital he reported that his breathing had returned to its previous normal pattern.

51. TWO NEW TOOLS FOR THE DIAGNOSIS OF ADRENAL CORTICAL DYSFUNCTION

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1. "Endogenous eosinopenia test." Samples of venous blood were withdrawn at 6:30 A.M. and at 9:30 A.M. of the same morning from eighty patients picked at random at the University of Minnesota Hospital. Breakfast was served at 7:30 A.M. A significant decrease in eosinophils from 6:30 to 9:30 A.M. was found in most of these patients. In patients with untreated Addison's disease or hypopituitarism, eosinopenia was not detected. This test is proposed as a new, simple, and harmless procedure for the detection of temporary or permanent states of adrenal cortical dysfunction. It is intended as part of an eosinophil test. The second part of this procedure (in essence a Thorn epinephrine test) would be carried out if there were no spontaneous eosinopenia. The second part is modified from the routinely used epinephrine test mainly by the injection of epinephrine immediately after the withdrawal of the first sample at 6:30 A.M. and by obtaining the second sample at 9:30 A.M. The extent and the regularity of the eosinopenia following either the subcutaneous injection of 0.3 mg. of epinephrine hydrochloride or the oral administration of ¾ grain of ephedrine sulfate at 6:30 A.M. will be compared with the hematologic effects of the same drugs at 8:00 A.M. The observation that spontaneous eosinopenia failed to appear in two patients with Cushing's syndrome and in certain other diseases (malignant hypertension, apoplexy, and porphyria) will be discussed.

2. A second test for the diagnosis of adrenal insufficiency depends on the assay of formaldehydogenic corticoids in urine after hydrolysis with calf spleen glucuronidase concentrate. The ratio of corticoids released by enzyme hydrolysis to corticoids released by acid hydrolysis (pH 1.0) ranged from 12 to 27, with an average of 18 in normal mature men and women. In five patients with Addison's disease and two patients with hypopituitarism the ratio was less than two in all instances. When standard procedures employed for estimation of urinary steroids yield inconclusive results, the determination of this ratio should be useful diagnostically.

52. THE EFFECT OF BENADRYL AND ACTH ON THE HYPOTENSION PRODUCED BY INTRAVENOUS HISTAMINE

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Our investigations into the effect of continuous intravenous infusions of histamine on coagulation phenomena and platelet activity on dogs led us into the field of antihistaminics. The data showing that Benadryl is ineffective in

preventing the clotting and platelet changes produced by intravenous histamine infusions are presented elsewhere.

Other investigators have reported the protection afforded by Benadryl against the fall in systolic blood pressure induced by single rapid intravenous injections of histamine. Our intravenous histamine infusions were given over a minimum period of ninety minutes and were necessarily of less strength than the single injections mentioned.

We have studied the systolic and diastolic blood pressures of three groups of dogs receiving large, continuous intravenous infusions of histamine over a ninety-minute period. The first group received no previous medication; the second was premedicated with 14 mg. per kilogram of Benadryl injected intraperitoneally one-half hour previously; and the third group was premedicated with 4.5 mg. per kilogram of ACTH injected intramuscularly twenty-four hours previously. No other studies were done on these animals, but all were kept for three weeks before they were studied, and were assumed to be free of serious endocrine pathology.

The systolic and diastolic blood pressures are presented as percentages of the initial readings for each animal, and these percentages are averaged for the entire group. In all groups the maximum drop in both systolic and diastolic arterial blood pressures occurred in twenty minutes.

Results (twenty minutes).—

Group I: Histamine alone. Systolic drop 39 per cent, diastolic drop 49 per cent.

Group II: Benadryl, then histamine. Systolic drop 14 per cent, diastolic drop 22 per cent.

Group III: ACTH, then histamine. Systolic drop 19 per cent, diastolic drop 64 per cent.

These results are interpreted to show that both Benadryl and ACTH protect the systolic arterial blood pressure against the profound fall produced in the normal dog by large, continuous injections of histamine. Benadryl appears also to prevent in large part the depression of the diastolic pressure, while ACTH seems to aggravate the depression of the diastolic arterial blood pressure.

53. FURTHER STUDIES OF INTRAVENOUS HISTAMINE ACTIVITY IN COAGULATION; IN VITRO AND BLOOD PRESSURE RELATIONSHIPS

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In previous investigations of the behavior of platelets in dogs receiving massive intravenous infusions of histamine, the circulating platelets were found to be reduced in number, and the clumping tendency of those remaining was increased. There were also a shortened clotting time, a marked drop in blood pressure, and an elevated hematocrit. These observations were made after ninety minutes of infusion.

In order to correlate the blood pressure changes with the above effect of histamine on platelets, the following experiments were performed.

In dogs given massive histamine infusions as before, maximum blood pressure drop was found to occur in twenty minutes. By this time the reduction in number and increased clumping of platelets, as well as decreased clotting time and increased hematocrit, had already occurred.

Aminophyllin administered intravenously at high concentrations produced hypotension of similar magnitude and rapidity as did histamine; but although a slight increase in hematocrit resulted after twenty minutes, there was no significant change in platelet activity or clotting time.

When Benadryl was injected intraperitoneally before intravenous histamine was begun, there was only a slight drop in blood pressure and little change in hematocrit, but platelet activity was increased and clotting time decreased as if no Benadryl had been given.

Preparations of platelet rich normal plasma prepared as in other experiments were divided into two parts. One was mixed with normal salt solution and the other with isotonic histamine diphosphate solution. Each was studied photographically under a wax seal for an hour. No qualitative or quantitative difference was noted in the platelets of the control or histamine samples.

Summary.—(a) The previously noted increase in platelet activity during massive intravenous histamine administration occurs rapidly and coincides with the time of the greatest blood pressure depression. (b) These changes are not the result of blood pressure depression or hemoconcentration. (c) Histamine has no effect on platelet activity in vitro. (d) Benadryl has no effect on the above-noted platelet and clotting time changes.

Conclusion.—Qualitative platelet activity increases rapidly when the concentration of blood histamine is increased, but is not related to blood pressure depression or the direct action of histamine on platelets.

54. INTRAPERITONEAL TRANSFUSION

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Blood has been injected intraperitoneally in lieu of the usual intravenous route for transfusion, although little is known about the efficiency of such a procedure. During the course of an experiment designed to study the symptoms produced by free blood in the peritoneal cavity, we had the opportunity to measure the results of transfusion by this method. Twelve subjects received from 115 to 650 ml. of blood in ACD solution, and one 500 ml. of unmodified blood by injection into the peritoneal cavity through the vaginal wall. Donor bloods were chosen which were compatible with that of the recipient, but serologically distinguishable. Erythrocyte counts of the subjects' peripheral blood were made by the Ashby technique to determine how many of the donor cells had gained access to the circulating blood. The greatest increment at twenty-four hours was 29,000 per cubic millimeter, and at forty-eight hours 53,300. The range was from zero to the latter figure. The mean increment at twenty-four hours was 10,800, and at forty-eight hours 22,880. When compared to intravenous injection the intraperitoneal administration of blood appears to be from 2 to 5 per cent efficient.

Further observations were made on seventeen subjects who received more than 400 ml. of whole blood intraperitoneally. Determination of serum chromogen contents of peripheral blood showed elevations at twenty-four and forty-eight hours, with mean values of 13.2 and 15.2 mg. per 100 ml., respectively. There were slight elevations of serum bilirubin in the same periods. The urea nitrogen levels were unaltered. Urines voided during the forty-eight hours after injection of blood contained no bilirubin and no increased amounts of urobilinogen.

Twenty-one subjects underwent laparotomy one to eight days after intraperitoneal transfusion, and the residual blood was collected from the peritoneal cavity. These bloods were found to be concentrated and to contain considerable free hemoglobin and bilirubin.

Conclusion.—Erythrocytes injected intraperitoneally did not appear in the peripheral circulation in significant numbers. The injected blood was slowly hemolyzed and slowly absorbed.

55. TWENTY-YEAR FOLLOW-UP STUDY ON PATIENTS INJECTED WITH RADIUM CHLORIDE

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The question of chronic human radium poisoning has not been settled in a quantitative sense due to (a) uncertainties in dosage received, (b) contamination of many past sources of radium with the shorter-lived mesothorium chain, and (c) frequent selection of cases on the basis of pathologic findings. The present study consists of a follow-up of a series of fourteen patients who received pure radium chloride intravenously twenty years ago.

These patients are the available residue of thirty-two inmates of the Elgin State Hospital who received between 70 and 440 μg of radium for therapeutic purposes in 10 μg weekly dosages. Their retention of the element up to twelve months after administration was reported by Schlundt and co-workers, and they have not been studied since 1933. The authors are grateful to Dr. D. L. Steinberg and other members of the staff of the Elgin State Hospital for making the patients available for study, and for their generous cooperation and assistance. Ten of the original patients are dead of various diseases apparently unrelated to the administration of radium.

Three of the patients showed a few localized areas of bone necrosis such as have been described in the previous literature. However, the majority of the patients showed minor, hitherto undescribed lesions in the cortices of the long bones, which we believe are also characteristics of ionizing radiation delivered to bone in situ by deposited radioelements.

Mean retention of the injected radium was 4 per cent at six months, 2.5 per cent at twelve months, and 0.8 per cent at twenty years. The present excretion pattern and ratio of exhaled radon to retained radium are similar to values reported by other investigators.

Aside from the changes noted in the skeleton, there were no clinical findings bearing any apparent relation to the radium injections. Careful hematologic studies have revealed no significant positive findings.

56. ELECTROCARDIOGRAPHIC CHANGES FOLLOWING CAROTID SINUS STIMULATION IN PAROXYSMAL SUPRAVENTRICULAR TACHYCARDIA

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Although the effectiveness of carotid sinus pressure (CSP) in terminating supraventricular tachycardia is well known, the method of conversion and the high incidence of heterotopic rhythms are not generally appreciated.

Continuous electrocardiograms were recorded during CSP in thirty-three attacks of supraventricular tachycardia occurring in twenty-two patients. Eighteen patients had one attack, two patients had two, one patient, five, and one patient six attacks. Conversion occurred in five general patterns: (a) asystole lasting up to six seconds, followed by normal sinus rhythm—twelve attacks; (6) slowing followed by sudden conversion—six attacks; (c) precipitous conversion—six attacks; (d) slowing, asystole, and then conversion—six attacks; and (e) ventricular tachycardia, prefibrillatory ventricular arrhythmia, lasting up to seven seconds, ventricular premature beats followed by normal sinus rhythm—six attacks. CSP had variable effects in the same patient during different attacks. The excitatory effect of carotid sinus pressure on stimulus formation in the heart was demonstrated by the high incidence of ventricular tachycardia (eight instances), and auricular, ventricular, and nodal premature beats (twelve instances). Changes in A-V conduction were demonstrated by the occurrence of varying degrees of A-V block. Intraventricular conduction was apparently altered also, as inferred from the occurrence of sinus rhythm with aberrant ventricular conduction after conversion, sinus rhythm with gradually changing intraventricular conduction while CSP was maintained, short P-R interval and aberrant or prolonged QRS complexes, and prolonged P-R interval and prolonged QRS complexes.

It is probable that the above effects of CSP are due to the release of acetylcholine within the heart. In addition to the usual muscarinic effect resulting in cardiac depression, the stimulating effect on the heart was manifested by the production of active ectopic beats in the atria and ventricles, arising in many foci, occurring singly, in bigeminy or trigeminy, and in prolonged bursts (prefibrillatory ventricular rhythms). The experimental production of similar changes by topical acetylcholine (Scherf and Chick) may support the above concept.

57. RESULTS OF AN INTEGRATIVE METHOD OF OCCUPATIONAL EVALUATION OF PERSONS WITH HEART DISEASE

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Successful employment of persons with heart disease depends upon selective placement. Unfortunately functional and therapeutic classifications, although valuable, are insufficient for practical job placement purposes. A clinic was established to study the problem of determining the employability of cardiac patients, and to match physical, vocational, and emotional aspects in individual cases to lead to gainful employment in competitive industry. The findings of cardiologist, medical social worker, and vocational counsellor were integrated in group conference. The value of this technique has been determined by a statistical analysis (IBM) of 150 adult patients studied in the past year.

Exclusive of patients in groups worse than III C, age, job opportunities, skill levels, literacy, emotional problems, and availability of company pension or insurance disability were greater deterrents to employment than the presence of heart disease. In a majority of cases, residual work potential was sufficient to allow the patients to return to their former occupation. Sheltered workshops and occupational therapy were valuable in restoring cardiac work capacity, and work motivation, especially in patients with significant organic

heart disease and iatrogenic cardiac disability. Our cardiac patients have been placed successfully in a variety of occupations, routeman, laborer, machine operator, etc. Their earnings have remained at the same or higher level than before the inception of their disease. In follow-up examinations every three months, there has been no evidence that the recommended employment has altered the physical status. The material accomplishments of the clinic include the following: help in obtaining initial employment or reassignment, vocational rehabilitation when necessary, improved work capacity, discovery of coexisting noncardiac disease, removal of erroneous label of heart disease in a few, and arousal of interest of labor and industry. Some interesting medical observations include the following: predominance of mesomorphs with excessive androgenic physical and mental attributes in the coronary group; the high incidence of ventricular aneurysms; the importance of the shoulder-hand syndrome; the adequacy of the coronary circulation in patients with old myocardial infarcts, as indicated by negative double Masters tests.

Our experience demonstrates that cardiac patients are not a serious risk if properly placed, and that they compete easily with the nonhandicapped worker.

58. TOTAL SERUM LIPID AND CHOLESTEROL LEVELS IN JAUNDICE

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A turbidometric method for the rapid estimation of the total lipid concentration in serum has been described by Kunkel, Ahrens, and Eisenmenger (*Gastroenterology* **11**: 499, 1948). Serum diluted with a reagent containing 1 per cent phenol and 12 per cent sodium chloride results in an even turbidity that can be readily measured. Values for normal individuals range from 16 to 30 units. The merits of this determination as an aid in the differential diagnosis of jaundice have been described.

This determination of the serum total lipid is technically much simpler than the determination of the total serum cholesterol. If the existence of hypo- or hypercholesterolemia can be thus predicted, its general usefulness as a screening test in clinical practice is obvious. In the present study, particular attention has been paid to its usefulness in patients with jaundice. The limitations of the serum cholesterol determinations as an aid in the differential diagnosis of jaundice are appreciated.

A simultaneous determination of the total lipid was made each time a serum cholesterol level was requested by the University of Minnesota Hospital laboratory service. Serum cholesterol values of 150 to 250 mg. per 100 c.c. are listed as normal in Table I. The results in 123 adult nonjaundiced patients (forty-eight had some evidence of liver dysfunction) are compared with eighty-six adult jaundiced patients.

Table I. Total Serum Cholesterol Levels

TOTAL LIPID LEVELS		123 NONJAUNDICED CASES			31 CASES OF SURGICAL JAUNDICE			55 CASES OF MEDICAL JAUNDICE		
		LOW	NORMAL	HIGH	LOW	NORMAL	HIGH	LOW	NORMAL	HIGH
	High	0	6	23	0	4	14	0	2	9
	Normal	5	51	5	1	8	0	8	15	1
	Low	23	10	0	2	2	0	19	1	0

The total lipid determination is useful in individual cases, especially when employed serially. It appeared to be relatively nonselective when employed as a screening test to detect hypocholesterolemia associated with liver dysfunction in instances of medical jaundice.

59. AN EXPERIMENTAL STUDY OF THE REACTION OF THE PULMONARY ARTERIES TO MINUTE EMBOLI

ROBERT B. JENNINGS, M.D. (BY INVITATION), BRYAN HUDSON, M.D. (BY INVITATION), AND WILLIAM B. WARTMAN, M.D., CHICAGO, ILL.

When minute emboli, having an average diameter of 0.5 mm., of rabbit blood, human fibrin, or cotton fibers were injected into the ear veins of healthy, adult rabbits they either became adherent to the walls of the pulmonary arteries or impacted in them. In either case an acute arteritis accompanied by endothelial proliferation regularly occurred. This process was reversible in some instances, but in others it was followed by organization of the emboli and eccentric fibroelastic thickening of the intima or bridgelike intravascular adhesions. Occasionally emboli adhered to the endocardium of the right ventricle and became organized there. Fatty degeneration, calcification, hemorrhage, and atheroma were absent, and the lesions appeared to be self-limited and to heal.

60. THE ARTERIAL PULSE WAVE

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The arterial pulse wave has been recorded from various parts of the body by a new device in normal subjects from the age of a full-term fetus during delivery to the age of 93 years, and from patients with various types of cardiovascular disease. The instrument has a low natural period, minimum overshooting, a lag of 0.004 second, and the record is calibrated to volume change and timed to 0.04 second. The components of the arterial pulse wave to be discussed are the first peak and notch, the second peak, the third notch and peak, the amplitude, the time of rise to the first peak or crest time, and the diastolic slope. The importance of variations from the normal in cardiovascular disease will be discussed. Lantern slides will be used to illustrate the discussion.

61. SERIAL OBSERVATIONS WITH THE LOW-FREQUENCY BALLISTOCARDIOGRAPH IN CONGESTIVE HEART FAILURE

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(INTRODUCED BY EMMET B. BAY, M.D.)

Serial observations with the low-frequency, critically damped ballistocardiograph on twenty-five patients treated for congestive heart failure were made before and after therapy, simultaneously with Benedict-Roth determinations of oxygen consumption. Certain patients were studied frequently throughout the course of their treatment.

The ballistic pattern was generally abnormal in congestive failure, but the only specific abnormality in form was an abnormally huge H wave, which

often dominated the complex and began its rising limb well before the onset of electrical systole as indicated by a simultaneously recorded electrocardiogram. This occurred in about 75 per cent of the cases and was uniformly reduced by therapy. Another characteristic finding, though not as exclusively limited to heart failure, was a reduction in the I-J amplitude or maximum systolic force, as measured in grams (F). With repair of congestive failure, F uniformly increased, and this was not offset by the slight reduction in pulse rate (P). Still more significant than the increase of F was the consistent reduction in the ratio of oxygen consumption to F × P, which has the same sense as the A-V oxygen difference.

Serial studies in six patients will be presented, in which particular attention is directed to the changes in I-J amplitude (F) with repair of congestive failure. With diuresis induced by digitalis or mercurial injections, there is a two- to threefold increase in F which is not usually sustained, but tends to fall back to an intermediate level of F sometimes no higher than the original value. The oxygen consumption falls during the period of diuresis, so that the ratio oxygen consumption to F × P is maintained at a reduced level. In a few patients in whom heart failure was first treated with bed rest or mercurials, the further administration of digitalis to the point of early toxicity caused no change in the ballistic form or amplitude. The interpretation of these findings and their clinical significance will be discussed.

62. CORRELATION OF CHEMICAL LIPID FRACTIONS OF BLOOD SERUM WITH GOFMAN'S MACROMOLECULES (Sf 10-20)

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(INTRODUCED BY EMMET B. BAY, M.D.)

Gofman has recently asserted that certain macromolecules, extracted from the blood serum by flotation and quantitated with the ultracentrifuge, represent a spilling over of lipids usually transported at lower density (Sf <10) in a loose combination with proteins. Chemical studies of the lipid fractions in this laboratory have demonstrated a significant difference between normals and myocardial infarction patients of comparable age in the per cent of serum lipid present as phospholipid, this value being lower in the infarction group. A comparison was made in a group of subjects between the milligram per cent of Sf 10-20, as measured by Dr. Gofman in his laboratory, and total lipids, phospholipids, and cholesterol determined on the same fasting serum by us (Bloor method). Correlations were then attempted.

Data were available on seventy serum specimens, drawn under fasting conditions from thirty-eight subjects, thirty-five of whom had sustained a myocardial infarction. The selection provided a wide range of values, even in individual cases contributing two or three samples. Correlation coefficients were not significantly different from zero between Sf 10-20 and total cholesterol, cholesterol/phospholipid ratio, or total phospholipid. There was, however, a significant correlation between Sf 10-20 and total lipid ($r = +0.395$) or per cent as phospholipid ($r = -0.455$). The correlation between per cent phospholipid and total lipid for these data was insignificant ($r = -0.129$). If partial correlations are calculated, holding total lipid constant, the relationship between Sf 10-20 and total phospholipid is improved ($r = -0.307$), while that between Sf 10-20 and per cent phospholipid remains significant ($r = -0.442$).

Gofman's macromolecules, then, correlate positively with total serum lipids and negatively with the per cent of serum lipid present as phospholipid

Insofar as phospholipids may represent ability to stabilize the serum lipids, it may be said that these data are not incompatible with the thesis that Gofman's macromolecules are a measure of the degree to which blood lipids are not adequately emulsified. Further, there is demonstrated a relationship between a chemical and a physical characteristic of the blood lipids, both of which are alleged to indicate atherogenesis, and may derive from the same metabolic defect.

63. REFEEDING (NUTRITIONAL) GYNECOMASTIA IN CIRRHOSIS OF THE LIVER

I. Clinical Observations

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In 1944 and 1945 transient gynecomastia and associated orchitis was observed in liberated prisoners of war in North Africa and the Far East. These bizarre signs invariably appeared in soldiers after refeeding and rehabilitation had begun to restore their severely wasted tissues. This type of gynecomastia, as far as is known, never appears in man or in male animals *during* the development or progression of natural or induced malnutrition.

In the past few years refeeding (nutritional) gynecomastia has been observed and studied in nine male patients ill with alcoholism, cirrhosis of the liver, and inanition. The transitory breast enlargements developed during rehabilitation and recovery on a high protein dietary regimen, and is different from the usual type of gynecomastia seen in cirrhosis, which is associated with feminization of the male and poor liver function.

Refeeding gynecomastia invariably appeared during remasculinization of the feminized cirrhotic and was associated with a gain in weight, slight testicular swelling, regrowth of male hair, improvement of liver function, and return of male libido. Prolonged metabolic balance studies were made in four of the nine patients, and these showed marked positive nitrogen balances and changes in electrolyte balances indicative of tissue regeneration.

64. RELATION BETWEEN SERUM AND HEPATIC ENZYMES IN EXPERIMENTAL LIVER DAMAGE

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Because determination of enzyme activity in the serum is commonly used to evaluate type and severity of liver damage, it seemed important to determine how accurately the serum enzymes reflect the function of the liver. Experimental liver damage of various types was, therefore, produced in rats and biochemical studies of liver homogenate, including esterase, alkaline phosphatase, choline dehydrogenase, and succinic acid dehydrogenase (tetrazolium reduction method) and histologic examination of the liver were correlated with serum esterase, alkaline phosphatase, and xanthine oxidase.

Chronic liver cell damage without marked regeneration (produced by a low protein diet or a normal diet with carbon tetrachloride, ethionine, or bromobenzene), was accompanied by a decrease of the oxidases and esterases and an increase of alkaline phosphatase of the liver homogenate. The serum

enzymes paralleled the liver enzymes consistently. In regeneration with hepatoma formation (produced by butter yellow) all studied hepatic and serum enzymes were markedly increased. The amount of enzymatic activity with the exception of alkaline phosphatase (which is increased also in damage) appears, therefore, to be correlated with cellular activity.

Acute liver injury was produced within forty-eight hours by carbon tetrachloride, bromobenzene, and ethionine. Ethionine, a methionine antagonist, causes a fatty liver without appreciable cell damage and, except for a decrease in liver choline oxidase, no enzymatic changes were found in liver or serum. Following carbon tetrachloride and bromobenzene which both produce marked liver cell damage, the hepatic enzymes were decreased. The serum, however, revealed dispersed results for esterase and xanthine oxidase ranging from decreased to increased amount. To clarify this discrepancy serum was obtained by serial heart punctures after carbon tetrachloride and bromobenzene administration. Esterase and xanthine oxidase increased consistently up to twelve to forty-eight hours, but dropped afterward below normal levels. This would indicate that the damaged liver releases its enzymes faster than normal and that decreased enzymatic activity in the serum becomes evident only after exhausting the liver. This explains the reliability of serum enzymatic activity for the indication of chronic liver damage and the variable levels of serum enzymes in acute injury, and should be taken into consideration in single determinations.

65. SPATIAL VECTORCARDIOGRAPHY: A MODIFICATION OF CURRENT CUBE METHODS OF RECORDING SPATIAL LOOPS AND A COMPARISON OF THE TWO TECHNIQUES

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(INTRODUCED BY WILLIAM MENNINGER, M.D.)

Spatial vectorcardiography is dependent upon the concept that the heart may be considered as a single dipole producing an electrical field in the body which acts as a homogenous volume conductor.

The spatial forces produced from ventricular excitation will describe a loop which has three dimensions.

The character of the loops have been shown to vary with the type of leads used to record the vectorcardiogram. The difficult task is to select a combination of leads which will accurately reproduce the loop as it is produced by the electromotive forces of the heart.

Because the oscillograph cathode ray tube enables one to use only leads which form right angles to one another, it has been necessary to select leads which are perpendicular. The most widely accepted technique has been the cube method which utilizes four electrodes geometrically equidistant from the electrical center. This technique has gained further favor by utilizing bipolar leads.

It was thought that the cube system did not give a true horizontal representation of electromotive forces due to the angle subtended to the electrical field by an electrode in the anterior and posterior right axillary lines. To circumvent this criticism we recorded the spatial vectorcardiogram by utilizing standard lead I, a vertical component by a negative electrode on the right arm and positive electrode in the right mid-axillary line at the level of the second lumbar vertebra, and a third lead consisting of a positive electrode on the back at the level of the second lumbar vertebra, and a negative electrode just above

the umbilicus. The angle subtended from the electrodes of the back and abdomen to the electrical field is quite different from that subtended by electrodes placed in the anterior and posterior axillary lines.

One hundred subjects have been investigated employing these two techniques and the results indicate that utilizing electrode placement as described above is superior to the usual technique employed by the cube method. The difference appears chiefly in recording the total electromotive force produced in an anterior posterior plane, rather than only a part of that force.

66. A RADIOCHEMICAL METHOD FOR THE DETERMINATION OF MICRO QUANTITIES OF THYROXIN

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(INTRODUCED BY OVID O. MEYER, M.D.)

A method for the quantitative analysis of thyroxin is presented, which employs an exchange reaction between elemental I^{131} and thyroxin bound I^{127}.

Solutions of known concentrations of thyroxin were incubated in the presence of I^{131}, and the rate of exchange between I^{131} and thyroxin I^{127} observed under varying conditions of pH, temperature, and concentration of elemental I^{127}. The thyroxin was removed from the incubation solution by extraction with butanol, and its radioactivity determined.

The optimal conditions for exchange during incubation have been determined. Under such conditions a direct proportionality has been demonstrated between the radioactivity of the butanol extract (thyroxin) and the concentration of thyroxin in the original solution.

The method has proved sensitive to quantities of thyroxin as small as 0.01 gamma.

It is believed that this method will be applicable to the determination of serum thyroxin levels, and other substances in which the exchange reaction is possible.

67. MEASUREMENT OF THE ANTIPYRINE SPACE IN EDEMATOUS STATES

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(INTRODUCED BY SMITH FREEMAN, M.D., PH.D.)

In normal adults and children, total body water as measured by the antipyrine space compares favorably with values obtained with deuterium. Little information is available on rates of equilibration or volumes of distribution of antipyrine in edematous states.

Antipyrine (1.2 to 2.0 Gm. in a 20 per cent aqueous solution) was injected intravenously into three groups of adult male patients: (1) normal subjects awaiting or convalescing from minor surgery, (2) cases of cirrhosis with ascites, and (3) cases of congestive heart failure with peripheral edema. Serial blood and edema fluid samples were drawn simultaneously two to eight hours following injections of the antipyrine.

Within eight hours, antipyrine failed to equilibrate completely between plasma water and ascitic fluid and/or between plasma water and peripheral edema fluid. Semilogarithmic plots of plasma antipyrine concentrations

against time yielded a linear relationship with the exception of the two-hour plasma values. These fell uniformly above the regression line, reflecting gross lack of mixing which was noted only in edematous patients. Blood samples drawn beyond four hours showed an exponential relationship with time. In the cirrhotic patients, if the regression line were fitted to include the aberrant two-hour blood concentrations, the calculated antipyrine spaces were on the average 2.8 ± 3.9 L. larger ($P = 0.04$) than if the two-hour value were excluded. In cardiac patients, inclusion of the two-hour blood level yielded values which were falsely high by a mean of 1.9 ± 3.0 L. ($P = 0.02$). In edematous states, it is recommended that blood samples be drawn at either 4-6-8 or 5-7-9 hours after the intravenous injection of 2.0 Gm. of antipyrine.

Mean antipyrine spaces in eleven normal subjects was 35.9 ± 4.9 L./1.73 m^2 or 52.5 ± 6.9 per cent body weight; in eleven cases of cirrhosis with ascites, 37.3 ± 3.1 L./1.73 m^2 or 52.7 ± 6.6 per cent body weight; in nine cases of congestive heart failure with edema, 37.8 ± 5.4 L./1.73 m^2 or 56.4 ± 8.3 per cent body weight. Marked overlap between antipyrine spaces in normal and edematous subjects emphasizes the need for serial determinations on a given subject rather than comparisons of group data.

68. THE DIURNAL AND NOCTURNAL VARIATION IN GASTRIC SECRETION IN MAN

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Quantitative measurements of the twenty-four hour gastric secretion in man and its diurnal and nocturnal variations have not been reported heretofore. The volume of the twenty-four hour continuous fasting secretion in normal individuals averaged 1,072 ml. as compared to 1,950 ml. in patients with duodenal ulcer and 1,048 ml. in patients with gastric ulcer. The output of free hydrochloric acid for patients with duodenal ulcer averaged 4,020 mg., as compared to 1,900 and 922 mg., respectively, for the normal and gastric ulcer groups.

Usually, the volume and output of acid during the day and night did not differ significantly in the normal group and in patients with gastric ulcer. In patients with duodenal ulcer, however, gastric secretion during the night significantly exceeded that produced during the day.

Gastric secretion was continuous in all three groups for the entire twenty-four hour period. The rate of secretion varied from hour to hour and usually followed a definite diurnal and nocturnal pattern. The secretion tended to be lowest between 5:30 A.M. and 11:30 A.M. The output of acid gradually increased after 11:30 A.M., the largest amounts being secreted between 5:30 P.M. and 11:30 P.M.; after 11:30 P.M. the secretion gradually decreased.

The secretion of acid was continuous only in the duodenal ulcer patients. In normal individuals and patients with gastric ulcer, the secretion of acid usually was intermittent with frequent periods of anacidity between 2:30 A.M. and 8:30 A.M. In practically all patients with duodenal ulcer the output of hydrochloric acid exceeded 50 mg. per hour throughout the entire twenty-four hour period; in more than half the group it consistently exceeded 100 mg. Persistent hypersecretion of this type rarely was encountered in normal individuals and patients with benign gastric ulcer.

(To be concluded in the December issue.)